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**THE PHOTOADAPTIVE RESPONSE  
OF  
HUMAN SKIN**

DR CAROL CHIU YEN OH ADIB

DOCTOR OF MEDICINE (MD)

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**The time course of photoadaptation and pigmentation studied using a novel method to distinguish pigmentation from erythema.**

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**Ultraviolet radiation sensitivity in vitiligo and adjacent normal skin.**

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### **List of Abbreviations**

NA	noradrenaline
MC1R	melanocortin-1-receptor
sem	standard error of the mean
UV	ultraviolet
UVA	ultraviolet A radiation
UVB	ultraviolet B radiation
UVR	ultraviolet radiation

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## Abstract

This thesis examines the photoadaptive response of human skin. Photoadaptation can be divided into two major components: pigmentation and epidermal thickening. These components safeguard the skin against future UV damage. This thesis is based on four main studies. The first study examines the photoadaptive response as a whole; this was found to be present over many weeks, decreasing to normal levels at approximately 10 weeks. The second study then examines the pigmentary component of the photoadaptive response. Following a single dose of UVB, pigmentation, measured by  $L^*$ , was maximal at Week 1; this pigmentary response subsequently decreased, however, was still present at Week 12. A pigmentary dose-response to UVB was clearly demonstrated. Subjects with red hair bearing homozygous or compound heterozygous MC1R mutations were also studied; these redheads pigmented to a lesser extent compared to non-redhead individuals; however, their rate of pigmentation loss was the same as non-redheaded individuals. The third study then examines the other component of the photoadaptive response: epidermal thickening, using UV Transmission Spectrophotometry. Following UVB exposure, there was an increase in skin darkness (measured by  $L^*$ ), melanosome density (eumelanin and pheomelanin) and epidermal thickness. The increase in thickness coincided with a greater level of photoprotection. The increase in epidermal thickness was greater for subjects of lighter constitutive pigmentation. The method of UV Transmission Spectrophotometry for measuring melanin density was found to correlate positively with a known chemical assay that measures eumelanin and pheomelanin. The fourth study examines the photoadaptive response of vitiligo skin. The melanin content of vitiligo skin was determined and found to be significantly reduced or absent. Given this, epidermal thickness is thought to be the main mechanism by which vitiligo skin photoadapts. This study found the epidermis to be thicker in areas of vitiligo. However, the greater epidermal thickness did not provide an equivalent level of photoprotection to normal pigmented skin. Having examined photoadaptation as a whole, and its two components separately, there is good evidence to support the fact that both, the pigmentary response and epidermal thickening, are important and contribute to towards the photoadaptive response as a whole.

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## Introduction

The human photoadaptive response can be defined as the mechanism by which human skin reacts to ongoing ultraviolet (UV) exposure. This mechanism serves for the skin to develop a tolerance to, and protect itself against further UV injury; repeated UV exposure leads to an increased tolerance to subsequent UVR-induced erythema<sup>1,2,3,4,5</sup>. In particular, UVB wavelengths are most effective in inducing a photoadaptive response, and this has been shown to be maximal at 1 week following exposure<sup>6</sup>. The cutaneous photoadaptive response varies from person to person, depending on various factors, such as pigmentation, body site, age, sex and environmental factors<sup>7</sup>. Two major factors contribute to the photoadaptive response as a whole: i) pigmentation or the production of melanin, and ii) epidermal thickening<sup>8,9</sup>. Melanogenesis is shown to account for only a component of the photoprotection induced by UVB; epidermal thickening, by indirect calculation, is thought to account for another component of the photoprotection<sup>10,11</sup>.

Cutaneous pigmentation increases following UV exposure; this is a mechanism by which human skin adapts to UVR<sup>12,13,14,15</sup>. Using a unique method of measuring pigmentation colour, the human pigmentary response will be examined in detail. Several studies into the time course of the pigmentary response have yielded differing results on when this response is maximal; the duration and peak of the pigmentary response is inconsistent amongst various studies<sup>12,13,14,15</sup>. The pigmentary response also depends on whether a single or multiple doses of UVR has been delivered, as each regime yields different results; it has been shown that a single dose of irradiation is more melanogenic than multiple fractionated doses<sup>16</sup>. Therefore, given the discrepancy in the literature, this study aims to map the time course of pigmentation as well as examine the pigmentary response in individuals of different constitutive skin colour and genotype. Melanogenesis occurs more readily in darker-skinned individuals than light-skinned individuals, for an equivalent dose of UV exposure (MED)<sup>17</sup>.

Two phenotypically distinct groups of people will also be studied in this thesis: individuals with red hair, and individuals of hair colour other than red. Redheads are phenotypically and genotypically distinct, with the key determinant of red hair colour being the Melanocortin-1-Receptor (MC1R) gene. The MC1R gene codes for a receptor which ultimately signals to increase the eumelanin:pheomelanin ratio<sup>18</sup>. Most individuals with red hair are homozygous or compound heterozygotes for MC1R variants, whereas non-redheaded individuals with pale skin are more likely to be heterozygotes<sup>19</sup>. The MC1R variants associated with red hair display a loss of function of the melanocortin-1-receptor, resulting in its inability to stimulate cAMP production as strongly as the wild-type receptor; this leads to lower eumelanin:pheomelanin ratios, and the red hair colour<sup>20</sup>. The rationale for comparing redheads with non-redheads was to prove the expectation that non-redheads possess a greater pigmentary potential, given the knowledge that redheads, by virtue of the loss of function mutation, lack to relative capacity to produce eumelanin. The rise in skin melanin content following UVR is shown to be due to eumelanin, and not pheomelanin<sup>21</sup>.

Epidermal hyperplasia is the other major component of the photoadaptive response<sup>22,23,24</sup>. Histologically, UV radiation induces hyperkeratosis, parakeratosis and acanthosis of the epidermis<sup>24</sup>. The method of spectrophotometry has been used for the evaluation skin colour and pigmentation<sup>25</sup>. Transmission spectrophotometry allows us to decipher the absorption spectrum of melanin, which falls within the ultraviolet and visible wavelengths<sup>26,27</sup>. From knowing the absorbance due to the epidermis and any scattering which takes place, it is then possible to calculate the thickness of the epidermis using a mathematical model<sup>28</sup>. This method has been used for calculation of epidermal thickness by other investigators; Bruls et al. found a good correlation between epidermal thickness measurements using transmission spectrophotometry and microscopy<sup>29</sup>. Certain investigators have not found UV sensitivity to be influenced by epidermal thickness<sup>30</sup>. Therefore, there is discrepancy in the literature on the matter of epidermal thickening as a response to UV exposure. This study, therefore, aims to clarify whether epidermal thickening is a response of the human skin following UV exposure. Using the methods of suction blistering and UV Transmission Spectrophotometry, experiments were performed which allowed for thickness comparisons of pre and post irradiated epidermis. Once again, subjects were of a variety of racial types, making it interesting when determining the melanin content of skin and drawing relationships between that and the epidermal thickness following UVR.

This thesis further investigates the situation in which epidermal hyperplasia is the only operable mechanism in adapting to UV exposure. In the skin condition of vitiligo, melanocytes are either decreased or absent; histopathologically, there are increased numbers of inflammatory cells and epidermal vacuolization with thickening of the basement membrane<sup>31,32,33,34</sup>. Given the lack of photoprotection due to melanin, we speculate that there must be an alternative mechanism by which vitiliginous skin gains photoprotection following UV exposure. Studies have already shown that there is less photoprotection in vitiligo; a lower dose of UVB is needed to produce the same level of erythema in vitiligo as in normal pigmented skin<sup>35</sup>. Vitiligo skin suffers greater UV injury following exposure to UVB<sup>36</sup>. UV-induced erythema and hyperkeratosis occur to a greater extent on vitiligo skin than on normal pigmented skin; this increase in thickness of the epidermis can be seen histologically<sup>37</sup>. In particular, the stratum corneum is thought to play a major role in photoprotection<sup>38</sup>. In studying the vitiligo skin of individuals, one realizes that both the vitiligo and normal pigmented skin on each subject would have undergone a similar number of years of exposure. Each would have been subjected to the same environmental factors and 'weathering', thus providing us with a picture of real life photoadaptation with and without melanin; it also provides information on the extent to which epidermal hyperplasia contributes to the whole photoadaptive response.

A description of the methods and instruments used in these experiments precedes the 'experimental studies' chapters. A novel method using iontophoresis of noradrenaline into human skin has been developed, and employed to enable non-invasive monitoring of skin pigmentation. This method is special in that it removes the influence of erythema or blood flow in the measurement of pigmentation

colour. The four 'experimental study' chapters that follow then lead us through a process in order to gain a better understanding of the human photoadaptive response.

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## Chapter 1 \* Materials & Methods

For all of the studies in this thesis, ethical approval was obtained through the Lothian Research Ethics Committee. All subjects were given a Patient Information Sheet regarding the study and what to expect during their participation; in addition, a verbal explanation of the study was also given. Written informed consent was then obtained by subjects signing a consent form prior to their participation (see Appendix); they were given the opportunity to discuss any aspect of the study with the researcher prior to, and during the course of the study. Subjects needed to have completed a questionnaire on entering the study (see Appendix). The inclusion and exclusion criteria for entering the study are detailed in Table 1 below.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none"><li>▪ Age 16 to 60</li><li>▪ Both sexes</li><li>▪ University staff</li><li>▪ General population</li></ul>	<ul style="list-style-type: none"><li>▪ Pregnant women</li><li>▪ History of phototherapy within the last 3 months</li><li>▪ Use of sunbeds or having had a sunny holiday within the last 3 months</li><li>▪ On photoreactive medication eg. minocycline, tetracycline, thiazides, amiodarone, chlorpromazine, ciprofloxacin.</li><li>▪ History of mental illness, dementia or on antidepressants</li><li>▪ On antihypertensive medication</li><li>▪ History of a pacemaker, angina or a myocardial infarct.</li><li>▪ History of thrombosis</li><li>▪ History of respiratory problems (emphysema/bronchitis)</li></ul>

Table 1.

### 1.1 Measuring Erythema with the Laser Doppler

Erythema was measured using a laser Doppler, which measures blood perfusion. The instrument used was the Flolab Laser Doppler (Moor Instruments, Devon, England). The instrument is sensitive to perfusion in blood vessels of less than 100 microns, including arterioles, venules and capillaries, and therefore is used for assessment of superficial microvascular blood flow<sup>1</sup>. The machine consists of an optical fibre probe which attaches to the skin surface. The emitted laser light is scattered by moving blood cells which impart a Doppler shift on the light. This is then processed to give a flux output or reading.

In this series of experiments, erythema was a result of UVB irradiation, and therefore needed quantification. Several studies along similar lines have relied on laser doppler flowmetry, reflectance spectroscopy and colourimetry in measuring UVB-induced erythema<sup>2, 3, 4</sup>. Both, reflectance spectroscopy and laser Doppler flowmetry, have been shown to be comparable in measuring

erythema<sup>5</sup>. The drawback of reflectance spectroscopy is that assessment of erythema is influenced by the presence of melanin in the skin and vice versa; light reflected from skin is analysed by the reflectance instrument within two bands of wavelengths centered at 546nm and 672nm, which are where oxygenized and deoxygenized haemoglobin absorb most strongly<sup>6</sup>. However, we need to bear in mind that melanin also absorbs wavelengths throughout these ranges, and therefore, will affect reflectance. The influence of melanin on measurements of erythema using reflectance is explained by the overlapping absorption spectra of melanin and haemoglobin. Melanin absorbs all wavelengths, albeit most strongly closer to the ultraviolet end of the spectrum. Haemoglobin absorbs specifically between wavelengths 540nm and 575nm<sup>7</sup>. Because of the overlapping spectra, the use of reflectance spectroscopy in measuring erythema cannot be without the influence of melanin. It has been shown that the perception of erythema is altered in heavily pigmented skin, such as Negro skin<sup>8</sup>; perception of erythema is related to reflectance, therefore, measurement of erythema using reflectance is not accurate in darker skin types. Laser Doppler flowmetry, however, allows for measurement of erythema without the influence of melanin and is the method used in this series of experiments. From hereon, the laser Doppler flowmetry technique will be referred to as the 'Laser Doppler'.

### **1.2 Measuring Pigmentation with the Minolta Chromameter**

Pigmentation was measured by reflectance colourimetry using the L\*a\*b\* system developed by the CIE<sup>9</sup>. The instrument used was the Chromameter CR-300 (Minolta, Osaka, Japan). This instrument consists of a xenon arc lamp housed within a chamber. The lamp produces diffuse and even lighting over an 8mm diameter measuring area which is applied flush to the skin. Light that is reflected perpendicular from the skin surface is then collected by an optical fibre cable for colour analysis. Colour is analysed using the L\*a\*b\* system. The L\* value measures colour on a black (0) to white scale (100). The a\* value measures colour on a red (+100) to green (-100) scale. The b\* value measures colour on a yellow (+100) to blue (-100) scale. This system possesses high reproducibility and is widely used for measurement of skin colour<sup>10</sup>. This instrument from hereon will be referred to as the 'Minolta Chromameter'.

It has been shown that total epidermal melanin content is the primary determinant of the L\* value, and that darker skin types have lower L\* values when compared with lighter skin types. The relationship between total melanin content and the L\* value is exponential, meaning that a small change in melanin content in lighter skin effects a large change in its L\* value<sup>11</sup>.

### **1.3 Iontophoresis of Noradrenaline**

The method of iontophoresis has been used in various studies for percutaneous drug delivery. Passive transdermal delivery of drugs occurs in small doses with non-ionized drugs<sup>12</sup>. Ionized drugs, however, do not penetrate the stratum corneum easily, and therefore, need an external source of energy to facilitate penetration into skin; iontophoresis aids this penetrations of charged drugs through skin<sup>13</sup>.

In addition, the flow of water from the skin surface during iontophoresis also aids drug delivery; in this manner, non-ionized drugs may also be delivered. Iontophoresis uses an electrical potential difference whereby charged ions in solution are driven into skin, the magnitude of the charge being dependent on the current and the duration of delivery<sup>14</sup>. Amongst the drugs delivered via this method are noradrenaline, acetylcholine, sodium nitroprusside and bretylium tosylate<sup>15, 16, 17, 18</sup>.

As described above, skin pigment colour measurements in these studies, were obtained using the L\*a\*b\* system. Although melanin is the major determinant of L\*, this measure becomes unreliable in vascular areas, and areas where there is inflammation and erythema<sup>19</sup>. Therefore, in these experiments, the method of iontophoresis was employed to eliminate the influence of erythema prior to measuring skin pigment colour. A vasoconstrictor, noradrenaline (1:1000, Abbott Laboratories Ltd, Kent, UK), was iontophoresed into skin areas prior to measurement of pigment colour. This produced temporary vasoconstriction of blood vessels, eliminating the influence of UV-induced erythema from true pigment colour measurement. The machine used was the Phoresor II Auto PM 850 (Iomed, Utah, USA) shown in Figure 1. It consists of a 9V battery-operated central unit with a positive and a negative electrode. A metal plate (Figure 2) attached to the positive electrode, was placed over noradrenaline-soaked cottonwool pads; these pads were strategically placed over the area of skin where colour measurements were to be obtained. A direct current of 0.5 mA was then run over a 5-10 minute period, iontophoresing NA through the skin. An alternative model, the PM700, is equipped with a Perspex well (Figure 3) which adheres to the skin with double-sided tape and conveniently holds the quantity of noradrenaline over the skin site to be iontophoresed. This technique of iontophoresis with noradrenaline was performed each time pigment colour measurements were to be taken over the course of the study.



Figure 1. The iontophoresis machine used for iontophoresis of noradrenaline through skin.

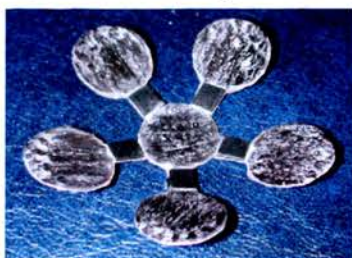


Figure 2. This metal plate is normally placed over noradrenaline-soaked cottonwool pads, strategically positioned over skin areas to be iontophoresed. This plate is attached to the positive terminal of the iontophoresis machine.





Figure 3. This Perspex well is an alternative to the metal plate. Only one area at a time can be iontophoresed with this well, into which, is dropped a 1 ml of noradrenaline.

The method used in these studies is as described. Firstly, flat round cotton wool pads of approximately 1cm diameter were placed over the areas to be blanched. Noradrenaline was then dropped onto these cottonwool pads, soaking them thoroughly. The strength of noradrenaline was of 0.1% or 1:1000. It requires approximately 0.5ml to soak one cottonwool pad. A metal plate (Figure 2) was then placed over these pads; the metal plate was connected to the phoresor machine (Figure 1). With the direct current set at 0.5mA, the length of time it takes to iontophorese noradrenaline to the point that there is satisfactory skin blanching is approximately 5 to 10 minutes. When iontophoresis of only a single spot is required, then a Perspex well (Figure 3) is used. The Perspex well is attached onto the skin surface with double-sided tape. Noradrenaline is then dripped into the central well – once again, approximately 0.5ml to 1ml is required. The direct current is then set at 0.5mA for 2 minutes.

In all cases, except for when there is intensely florid erythema, this method was successful in producing sufficient blanching of the skin. Sufficient blanching is confirmed by a Laser Doppler reading that is comparable with a reading obtained when blood flow to one's arm is restricted by a blood pressure cuff. (The arm would be elevated and milked off blood prior to applying the cuff.) In such a case of a blood pressure cuff being applied to the arm above the systolic pressure, there is loss of circulating blood flow, therefore a reading by the Laser Doppler in this situation is due to Brownian motion. It was found that when Laser Doppler readings were taken following iontophoresis with noradrenaline, readings were similar to when a blood pressure cuff was applied to the arm, confirming satisfactory cessation of circulating blood flow.

The only problem faced by noradrenaline iontophoresis was when erythema was so intense that blanching could not be achieved. In hindsight, this could perhaps have been helped by increasing the dose or strength of the noradrenaline solution (greater than 0.1%); varying the time and the current may also have helped. A second cycle of iontophoresing could have been tried in the attempt to achieve blanching of intensely florid erythema. A literature search found no reports on iontophoresis of noradrenaline for the purpose of inducing skin blanching; this is a new method and therefore will need further fine-tuning in the future. The metal plate (Figure 2) was self-improvised to suit the pattern of irradiated spots on the subjects' skin – this could have been helped by a more accurate device that

could deliver iontophoresis to all 6 areas simultaneously. Perhaps multiple perspex wells would have been a neater way of iontophoresing multiple sites.

## 1.4 UVB Irradiation

### 1.4.1 Waldmann UV 801

This will be referred to as the *Waldmann TL01 Lamp*. This UVB irradiation source was used in the Photoadaptation Study (Chapter 2). It was manufactured by Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen. This machine was factory-fitted with TL01 lamps and emitted wavelengths in the narrowband UVB spectrum peaking at 311 nm (Figure 4). It was useful for irradiating large areas such as the back; any smaller areas were irradiated using a template cut to size and shape.

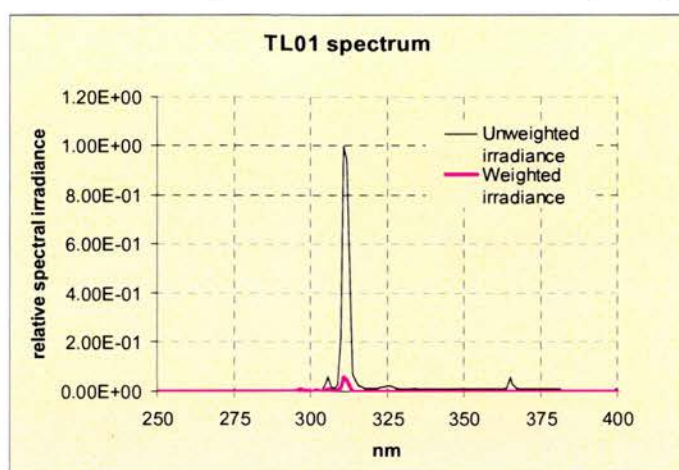


Figure 4. Emission spectrum of the Waldmann TL01 Lamp used in these experiments, as measured using a spectroradiometer <sup>20</sup>.

### 1.4.2 Diffey Wideband UVB Phototesting Unit (Diffey Lamp)

This machine was specially built by Professor Brian Diffey (Medical Physics, University of Newcastle, UK) and will be referred to as the *Diffey Lamp* from hereon. A similar machine based on this design has been used for phototesting to ascertain the MED prior to phototherapy (for psoriasis/atopic dermatitis)<sup>21</sup>. The machine used in the following studies contains one Philips TL12 broadband UVB fluorescent lamp. It delivers 10 fixed doses via a handheld wand.

The doses are: 300 mJ/cm<sup>2</sup>, 238 mJ/cm<sup>2</sup>, 189 mJ/cm<sup>2</sup>, 150 mJ/cm<sup>2</sup>, 119 mJ/cm<sup>2</sup>, 95 mJ/cm<sup>2</sup>, 75 mJ/cm<sup>2</sup>, 60 mJ/cm<sup>2</sup>, 47 mJ/cm<sup>2</sup> and 38 mJ/cm<sup>2</sup>. When switched on, it illuminates for a fixed 30 seconds, delivering the range of doses over 10 apertures of 0.8 cm diameter each. Over each aperture is a metal grating with perforations. The larger the perforations for an aperture, the greater is the dose delivered through that aperture; likewise, the finer the perforations, the more attenuated is the dose delivered.

The emission spectrum of this lamp is shown in Figure 5. The Diffey Lamp was used in the Photoadaptation Study (Chapter 2) and the Epidermal Thickness & Melanin Study (Chapter 4).



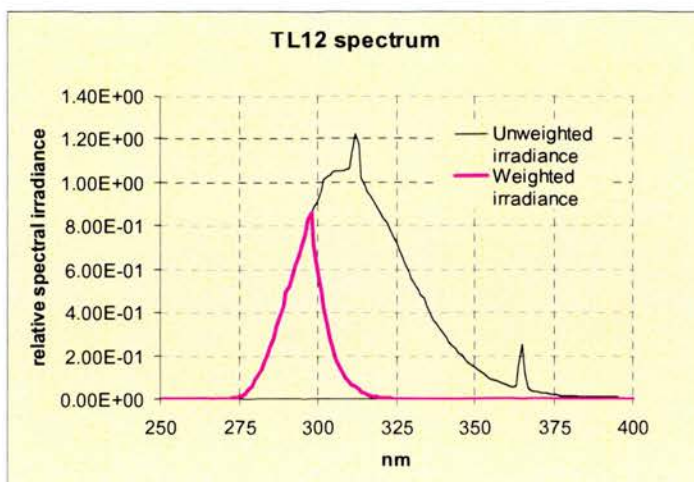


Figure 5. Emission spectrum of the Diffey Lamp used in the experiments, measured using a spectroradiometer<sup>22</sup>. The unweighted irradiance refers to absolute irradiance; the weighted irradiance is the absolute irradiance multiplied at each wavelength by the skin erythral weighting factor.

### 1.4.3 Oriel Xenon Arc Lamp

This UVB source was the Oriel Model 68811 used with a 300W Xenon Arc Lamp Model 6258 (Oriel, Stratford, California), optically coupled to an interference filter (Andover 300FS10-50 AM-33230-01; peak transmittance 300nm). This allowed UVB radiation mainly in the range of 275-325nm with a central wavelength of 300nm (Figure 6). This machine was used to irradiate skin at various doses ranging from 28mJ/cm<sup>2</sup> to 160mJ/cm<sup>2</sup> in the Pigmentation Study (Chapter 3). An optical fibre cable attached to the arc lamp housing enabled versatility in irradiating 1cm<sup>2</sup> areas of skin. This machine will be referred to as the *Xenon Arc Lamp* from hereon.

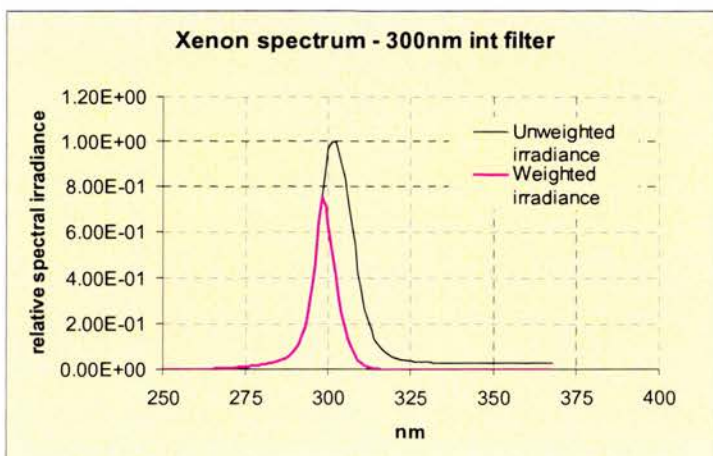


Figure 6. Emission spectrum of the Xenon Arc Lamp used in experiments, as measured by a spectroradiometer<sup>23</sup>.

### **1.5 Suction Blistering**

In the Epidermal Thickness & Melanin Study (Chapter 4) and the Vitiligo Study (Chapter 5), suction blisters were created on the skin of human volunteers. The purpose of creating suction blisters was to obtain a sample of full thickness epidermis; this was necessary in order for its thickness to be measured and its melanin content quantified via the method of absorbance spectrophotometry (described later).

The method of suction blistering has been used for various purposes, such as skin grafting of wounds and correcting areas of vitiligo<sup>24, 25</sup>. Suction blistering cleaves the skin at the dermo-epidermal junction, such that the roof of the blister is the entire full thickness epidermis. This is proven by the demonstration of bullous pemphigoid antigen on the epidermal side of the separation<sup>26, 27</sup>. The separation occurs above the lamina lucida; the epidermal cells are vital for 4 to 5 days post blistering<sup>28</sup>.

The variables in blister formation are: suction pressure and blister time:

$$\text{Suction pressure} \times \text{Blister time} = \text{Constant}^{29}$$

The Constant varies depending on body site and person; there is an individual variation of 30%. Suction pressures of 150-200mmHg have been shown to create a blister within 3 hours; higher suction pressures of 250-350mmHg, although quicker, result in bruising and pain<sup>30</sup>. Suction pressures of 300mmHg have been used to produce blisters in 1 to 2 hours using a the large end of 10 or 20ml syringe barrels<sup>31</sup>. Blister formation is faster at higher skin temperatures<sup>32</sup> and in older subjects; in young adults, females are quicker to blister than males<sup>33</sup>. There is no effect of blood flow on blister formation. For blister diameters of 0.5-2cm, the diameter of the blister does not affect the time to blistering<sup>34, 35</sup>.

In the following experiments, suction blisters were created using a device called PTC 3300 VAC Vacuum Unit (InnoKas Medical Oy, Finland) shown in Figure 7. This machine creates a negative suction pressure of up to 400mBar (300mmHg), which can be applied to the skin, to create a blister. The base of a 5ml syringe barrel (plunger removed) was used to create a blister of 1 cm in diameter (Figure 8). The head of the syringe was connected to a rubber tubing, attached to the central vacuum pump machine. When the machine was turned on, suction was created via the tubing and syringe barrel onto the skin surface. The barrel was taped in place and within 1 to 3 hours, a suction blister was produced (Figure 9).





Figure 7. The machine for creating suction blisters.



Figure 8. Creating a suction blister.



Figure 9. A formed blister after 1-3 hours of suctioning.

### 1.6 Transmission Spectrophotometry

Transmission spectrophotometry is used in Chapter 5 to determine the epidermal thickness and melanin concentrations of epidermal samples obtained by suction blistering. The background literature behind this technique is explained further in the relevant chapter.

The machine used for transmission spectrophotometry was the Fluoromax-3 Spectrophotometer (HORIBA, Jobin Yvon Inc, New Jersey USA), shown in Figure 10. This machine allows epidermal samples to be placed in the optical path of an ultraviolet spectrum (250-400nm). This process occurs within an integrating sphere which collects transmission of all wavelengths travelling through the sample. In order to avoid shrinkage, the epidermal samples were placed in 0.9% normal saline, in quartz cuvettes (Figure 11) before being placed into the integrating sphere within the spectrophotometer. The transmission of the wavelengths through each sample is measured by the machine in conjunction with accompanying Datamax software. The transmission profile is, of course, related to the absorbance profile of the epidermal sample, from which can be calculated the absorbances due to: i) epidermal proteins (indicative of thickness), and ii) melanin. The mathematical model used for these calculations demonstrates that the spectral absorbance of epidermis is the sum of the individual absorbances: absorbance due biomolecules such as amino acids and nucleic acids, absorbance due to melanin, and scattering within the epidermis<sup>36</sup>. From these absorbances, are derived epidermal thickness and melanin density.

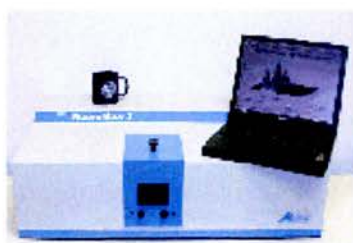


Figure 10. The spectrophotometer used to determine absorbance of suction blistered epidermal samples.



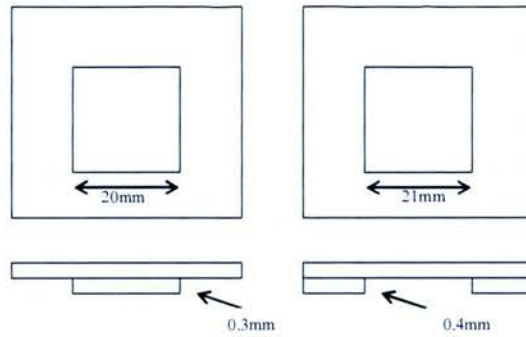


Figure 11. The quartz cuvette used to contain the epidermal sample shown as the opaque disc in the centre of the cuvette.

### 1.7 Statistical Analysis

Statistical analysis was done using Microsoft Excel software. Paired t-tests were used for comparison of data throughout the studies: changes in flux, changes in pigmentation colour, the difference between redheads and non-redheads, as well as between Caucasians and Asians, changes in epidermal thickness, changes in melanosome density, and changes in eumelanin and pheomelanin concentrations.

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## Chapter 2 \* Photoadaptation Study

### 2.1 Introduction

The purpose of this experiment was to study the human photoadaptive response. The question we would like to ask is: does prior exposure to UVR lend tolerance to the skin against future exposures? In other words, is there an adaptive mechanism which protects the skin from future UVR damage. This concept of adaptation to UVR can be variously called photoadaptation, photoprotection, tolerance, accommodation, acclimatization or reduced sensitivity. If there is a photoadaptive response, it is helpful to know when it peaks or is maximal, and also the duration of this response.

It has been shown in several studies that repeated skin exposure to UVR leads to an increased tolerance to subsequent UVR-induced erythema<sup>1,2,3,4,5</sup>. This photoadaptive response helps in protecting the skin from further UVR-induced damage<sup>6</sup>. The components of the photoadaptive response have been studied, and it is thought that pigmentation, as well as an increase in epidermal thickness, both, play important roles in protecting the skin from further UV-induced DNA damage<sup>7,8</sup>. The tan gained from UVA and UVB irradiation are shown to provide similar levels of protection against DNA damage (by numbers of endonuclease-sensitive sites in biopsies), however, a UVB tan provides greater protection against erythema than a UVA tan; this suggests another mechanism of protection induced by UVB, such as epidermal thickening<sup>9,10</sup>. UVB wavelengths are shown to be most effective in inducing protection/adaptation, and this photoadaptive response is shown to reach a maximum at seven days<sup>11</sup>. Melanogenesis is shown to account for only a component (6-11%) of the photoprotection induced by UVB; epidermal thickening, by indirect calculation, is thought to account for another component of the photoprotection<sup>12,13</sup>. The concept of photoadaptation as a whole, comprising pigmentation by melanogenesis, and epidermal thickening, is certainly a plausible one. Therefore in this experiment, the aim is to measure and prove the existence of photoadaptation 'as a whole'. The next two chapters will tease apart the components of photoadaptation, measuring each of them separately: Chapter 3 will isolate and measure the component of **pigmentation** in the photoadaptive response, and Chapter 4 will isolate the component of **epidermal thickening** by direct measurements.

In measuring photoadaptation as a whole, this study is divided into two phases: 1) The Photoadaptive Phase and, 2) The Challenge Phase. In the Photoadaptive Phase, an area of skin on the subject was given UVB radiation in the attempt to promote an 'adapted' area of skin which was to be challenged at a later time with higher doses of UVB. The degree of adaptation gained by that patch of skin from its prior exposure can then be measured by comparing the erythema produced in that patch of skin, and the erythema produced on a normal unadapted patch of skin.

In the Challenge Phase, three different doses of UVB were irradiated onto the previously adapted patch (as mentioned above), and onto an adjacent patch of control unadapted skin. DNA is a chromophore

for erythema, and it has been shown that DNA photodamage is related to the physical dose of UVR received<sup>14</sup>; therefore in this experiment, 3 different challenge doses of UVR were used to produce different levels of erythema, demonstrating a dose-response.

## 2.2 Methods

The demographic data on the eleven subjects participating in this study is detailed in Table 1.

Subjects	Age	Sex	Fitzpatrick Phototype <sup>15</sup>	Hair Colour
1	45	F	2	Blond
2	29	M	2	Brown
3	48	M	2	Brown
4	18	F	2	Brown
5	49	F	2	Brunette
6	49	F	2	Blond
7	24	F	2	Blond
8	33	F	2	Brown
9	55	F	2	Brunette
10	36	F	1	Blond
11	53	F	2	Brunette

Table 1. Subject details

### 2.2.1 Adaptation Phase

Eleven healthy subjects, consisting of 2 males and 9 females, were recruited for this study (detailed in Table 1 above). In order to promote a photoadaptive response on the right lower back of each subject, they each received 3 doses of UVB on the right lower back over 3 consecutive days (one dose per day). The irradiated area was a square of 9cm x 9cm. The UVB source was the Waldmann UV 801 TL-01 (Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen, Germany). The 3 doses delivered over 3 consecutive days were 480mJ/cm<sup>2</sup> (2.7 SED), 520mJ/cm<sup>2</sup> (2.9 SED) and 570mJ/cm<sup>2</sup> (3.2 SED); these were typical doses used in phototherapy for psoriasis. The adjacent left lower back was left unirradiated, serving as the unadapted control patch of skin. The timing of events is detailed in Figure 1 below.

### 2.2.2 Challenge Phase

Following the last day of the Adaptation Phase, the adapted square on the right lower back of each subject was challenged at several time points: ½, 1, 2, 3-4 and 10-12 weeks. At each time point over the next 10-12 weeks, the square was challenged with 3 UVB doses delivered alongside one another: 189mJ/cm<sup>2</sup> (5.2 SED), 300mJ/cm<sup>2</sup> (8.2 SED) and 476mJ/cm<sup>2</sup> (13 SED) (see Figure 2). The doses were

delivered in small spots of 0.8 cm in diameter using the Diffey Lamp. Not only were the 3 doses administered to the adapted square on the right lower back, but also to the unadapted skin of the adjacent left lower back which served as a control. Photoadaptation was defined by the difference in erythral response between the adapted and unadapted areas following the standard challenge doses.

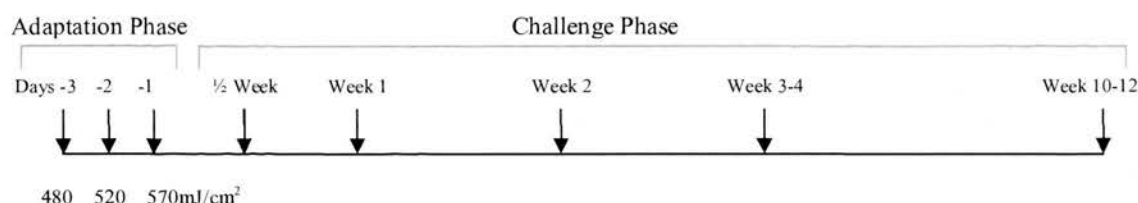


Figure 1. Time-line of events showing timing of the Adaptation Phase and Challenge Phase.

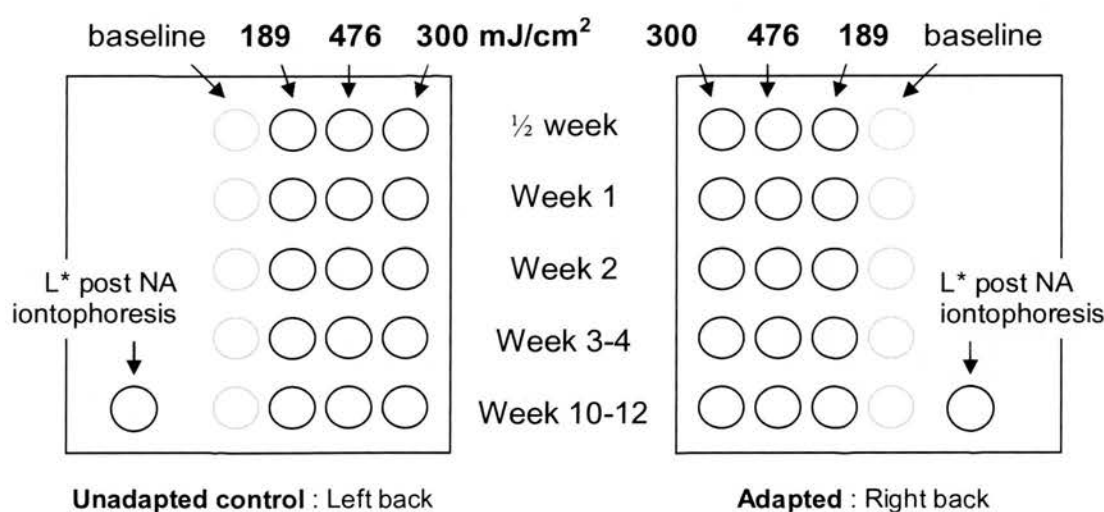


Figure 2. Diagram of the back of a typical subject with the control (left) and irradiated patch (right) being challenged by the specified doses.

It was for pragmatic reasons that the narrowband Waldmann TL-01 lamp was used in the Adaptation Phase, and the wideband Diffey Lamp in the Challenge Phase. Because the Waldmann TL-01 has a large irradiating surface, it was suited to photoadapting large areas of skin as was called for in this study. The Diffey Lamp was used in the Challenge Phase as the design of the lamp made it convenient to give three different doses simultaneously within close proximity of one another. The choice of lamps was also limited to what was available in our research laboratory.

### **2.2.3 Measurement of erythema and pigment colour**

Twenty-four hours following each challenge, the erythema of the 3 challenged spots on the adapted and unadapted sites were measured using the Laser Doppler; measurements were taken in triplicate and a mean of the three readings was calculated. For both, the adapted right back and the unadapted left back, a baseline reading was taken on adjacent unchallenged skin. This serves to provide information on baseline flux for the adapted and unadapted sites. The mean increase in flux can then be ascertained for each site (adapted and unadapted) by subtracting the baseline flux from the mean flux of the respective challenged spot. The mean flux induced by the challenge on the adapted skin was then compared with that of unadapted skin.

Skin pigmentation of both the adapted and control unadapted skin was also measured by the Minolta Chromameter using the L\*a\*b\* system. In order to obtain the true pigment colour that is uninfluenced by erythema, the method of noradrenaline iontophoresis was employed (described in Chapter 1). This technique was performed at each time point over the course of the study.

## **2.3 Results**

### **The Flux of adapted and unadapted skin following challenge**

Following each challenge with UVB doses of 189mJ/cm<sup>2</sup> (5.2 SED), 300mJ/cm<sup>2</sup> (8.2 SED) and 476mJ/cm<sup>2</sup> (13 SED), the mean increase in flux of the adapted site was compared with that of the unadapted site. Figure 3 illustrates that on almost every occasion, the mean increase in flux of the adapted site was less than that of the unadapted site. This was consistently the case for Weeks ½, 1, 2 and 3-4, with a significant difference between the adapted and unadapted sites ( $p < 0.05$ ). By Week 10, the difference between sites was no longer significant, except for the lowest dose of 189mJ/cm<sup>2</sup> (5.2 SED) ( $p = 0.03$ ). Table 2 shows the results in a tabulated format. Multiple paired t-tests were used here because the studies were pre-planned that all doses would be examined, and no formal allowance was made for multiple testing, as there was a strong prior biological hypothesis. The alternative of a repeated measures ANOVA, or a split plot analysis was considered, but t-tests, although much simpler, were felt to provide illustrative guides to significance, since we were only interested in consistent differences between groups. This was performed on advice of statistical advisor, Professor Jonathan L Rees.



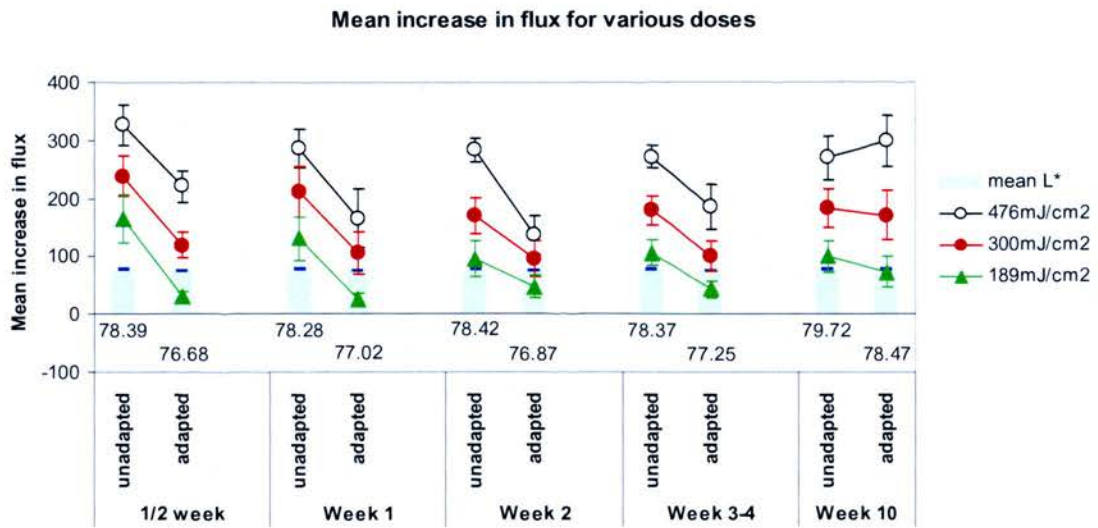


Figure 3. The mean increase in flux of the adapted site was consistently less than that of the unadapted site generally throughout the course of the study (except for Week 10). The mean L\* values for the adapted and unadapted sites are also shown as the light blue bars, with actual values specified below each bar. The mean L\* value of the adapted sites are lower (darker) than that of the unadapted site.

Mean increase in Doppler Flux (± SEM)							
		476mJ/cm²		300mJ/cm2		189mJ/cm2	
1/2 week	Unadapted	327.61	(34.73)	238.73	(34.48)	165.85	(40.44)
	Adapted	220.88	(28.33)	120.06	(21.22)	31.18	(6.71)
Week 1	Unadapted	286.21	(34.06)	212.85	(44.09)	131.06	(37.43)
	Adapted	166.64	(49.66)	106.61	(35.43)	27.3	(9.77)
Week 2	Unadapted	283.97	(19.44)	170.79	(30.52)	95.73	(31.39)
	Adapted	137.24	(33.81)	95.88	(32.00)	47.15	(19.22)
Week 3-4	Unadapted	272.30	(20.27)	180.18	(24.33)	107.39	(20.75)
	Adapted	186.85	(38.71)	100.58	(25.33)	43.18	(13.16)
Week 10	Unadapted	270.15	(37.72)	183.57	(33.19)	100.18	(27.63)
	Adapted	299.30	(43.74)	170.85	(42.73)	73.7	(26.66)

Table 2. The mean increase in flux of both unadapted and adapted sites.

The diagram below (Figure 4) shows the results of individuals following the three different challenge doses. Of the 11 individuals, one was a redhead. Because there was only one redheaded individual, a comparison cannot be made between redheads and non-redheads with regard to their photoadaptive response. The topic of comparing redheads and non-redheads and the rationale behind it will be discussed further in Chapter 3.

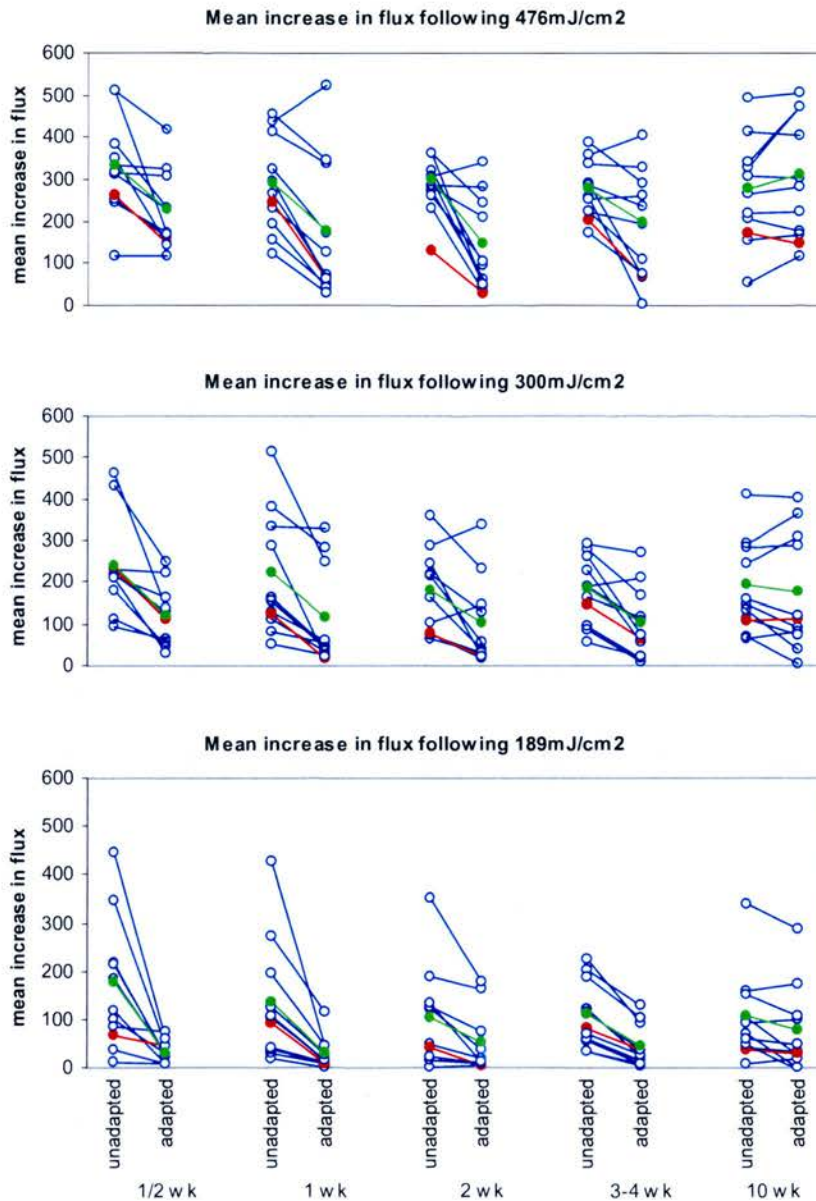


Figure 4. Individual results are shown for the mean increase in flux of the adapted and unadapted sites following the three different challenge doses. The red circle is a redheaded individual and the blue circles are non-redheads; the green circle is the mean of all the non-redheads.

The graph, Figure 5, further investigates whether there is a relationship between the constitutive pigmentation of the individuals and how much flux (erythema) is generated following a challenge with the highest dose of  $476\text{mJ}/\text{cm}^2$ . Data are plotted for: i) flux generated on the unadapted area, ii) flux generated on the adapted area and, iii) the difference in flux between the unadapted and adapted areas. The results show that there was no relationship between constitutive pigmentation and the amount of flux produced following a UV challenge, whether it was on unadapted or adapted skin.

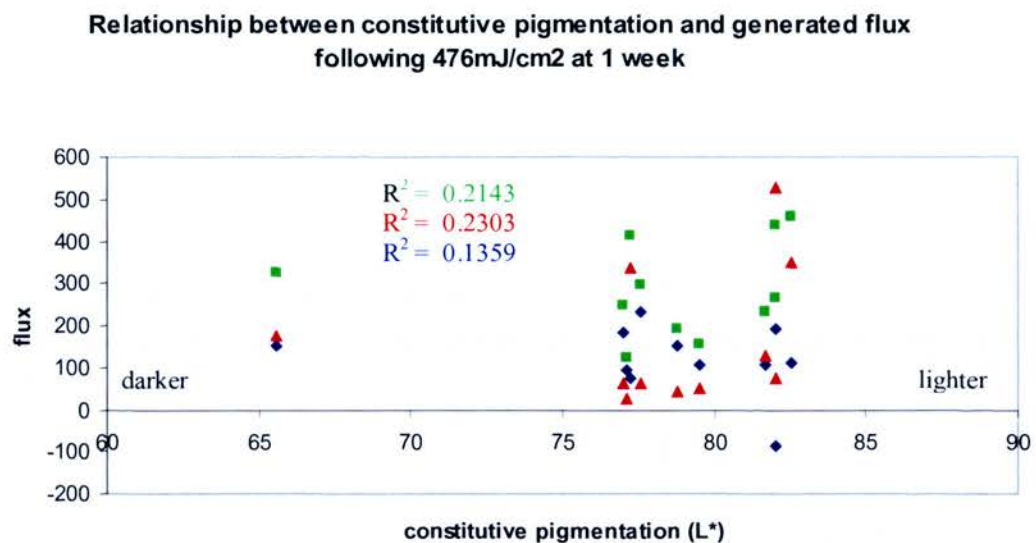


Figure 5. This graph shows that there is no relationship between constitutive pigmentation and the amount of flux generated on the individual subjects following a challenge with  $476\text{mJ}/\text{cm}^2$  at 1 week.

- Green squares indicate flux of unadapted areas following the challenge
- Red triangles indicate flux of adapted areas following the challenge
- Blue diamonds indicate the difference in flux between the unadapted and adapted areas.

$R^2$  values are provided for trendlines for the three different sets of data (trendlines omitted for sake of visual appearance); they show poor correlation between constitutive pigmentation and the amount of flux generated.

## 2.4 Discussion

In this experiment, the photoadaptive response was examined in its entirety. The study shows that several doses of UVB radiation are capable of initiating an adaptive response in human skin. In this study, erythema was used as a measure of UV damage<sup>12</sup>; it was clearly shown that when challenged with UVB radiation, the photoadapted area of skin produced less erythema, compared with normal unadapted skin. It was shown that the photoadapted square of skin became more pigmented following the Adaptation Phase. We can theorize that this increase in pigmentation contributes to the photoadaptive response; this theory will be further investigated in next chapter. Whether there is



another component contributing to the photoadaptive mechanism as a whole, is of particular interest, and will also be investigated in the later chapters.

The time-scale chosen for this experiment showed that by Week 10, the photoadaptive response was no longer present (except for the lowest challenge dose used). Unfortunately, due to the lack of measurements between Week 4 and Week 10, we are unable to chart the slow diminution of the photoadaptive response. Interestingly, the photoadaptive response was still evident following the challenge with the lowest dose ( $189\text{mJ/cm}^2$ ) at Week 10, but not evident following a higher dose challenge ( $300\text{mJ/cm}^2$ ). Perhaps, as the photoadaptive response wears off with time, it can only withstand challenges of progressively lower doses of UVR. In other words, when the photoadaptive response is waning (after a long duration from the time of adaptation), only progressively lower challenge doses continue to be protected by the photoadapted area of skin. An improvement to this study would be to continue to challenge both sites with progressively lower doses for a longer time period of time.

The UVB doses used in the Adaptation Phase were chosen as they were typical of doses used for patients undergoing phototherapy treatment (mainly for psoriasis):  $480\text{mJ/cm}^2$  (2.7SED),  $520\text{mJ/cm}^2$  (2.9SED) and  $570\text{mJ/cm}^2$  (3.2SED). This is relevant in the situation where phototherapy regimes are interrupted by unforeseen circumstances; in such a scenario, it is helpful to know, if after a period of time away from their phototherapy regime, these patients still maintain a level of photoprotection enabling them to resume the regime without having to re-commence at a lower dose. From the results of this experiment, it is evident that photoprotection is maintained for at least 3-4 weeks. However, it is not clear if this is true for higher challenge doses than used in this experiment ( $> 476\text{mJ/cm}^2$  or 13SED). A future study with a wider range of challenge doses ( $<189\text{mJ/cm}^2$  or 5.2SED and  $>476\text{mJ/cm}^2$  or 13SED) would be valuable in answering this question.

The standard erythema dose (SED) of the challenge doses used (5.2 SED, 8.2 SED and 13 SED), are also relevant to ambient sunlight exposure in summer at various parts of the world. For example the standard erythema dose for a person who would have spent from sunrise to 12:30 midday in July at 50 degrees latitude North (eg. London) is 5.04 SED; spending an equivalent time of half a summer's day in the southern hemisphere at 30 degrees latitude South (eg. Brisbane, Australia) in January from dawn until 12:30 midday is 9 SED. Therefore, the lowest challenge dose of 5.2 SED is equivalent to half a day's exposure on a summer's day in London and the middle challenge dose of 8.2 SED is equivalent to half a day's exposure in summer in Brisbane, Australia.

The racial mix of the subjects in the study group was of British Anglo-Saxon descent. They were of skin Phototypes I-II. Unfortunately, this study group does not represent the diversity of our world population. Therefore these results only apply to the white Anglo-Saxon population of skin Phototypes I-II. It is known that there is a difference in the photoprotective qualities of black and white skin, with

black epidermis being three to four times more photoprotective than white epidermis at all wavelengths<sup>16</sup>. Most of the photoprotective property of black skin, compared to white, is derived from melanosomes in the malphigian layer; the photoprotection afforded by the stratum corneum is only slight<sup>17</sup>. Stratum corneum thickness has not been found to be significantly different between European and African subjects<sup>18,19</sup>. Therefore, given this difference in the photoprotective qualities of black and white skin, it would be valuable to study and investigate the photoadaptive response of darker racial types. It has been shown that, after a similar level of UVB-induced erythema, more melanogenesis occurs in subjects with darker skin than in subjects of lighter skin<sup>20</sup>. Constitutive skin colour itself, has a role in photoprotection, with darker constitutive colours demonstrating better photoprotection and less DNA damage following UVR<sup>21</sup>. Future studies building on our present study, need to be undertaken whereby the range of skin Phototypes I-IV, are used. In such a study, I suspect that the Adaptation Doses and Challenge Doses used for darker subjects may need to be different to those used in the present study, in order to achieve meaningful results. The duration of the photoadaptive response in darker subjects should also be compared to that of lighter skinned subjects. It would be interesting to observe if darker subjects are able to maintain their photoadaptive response for a longer period of time for: i) a given dose of UVR, and, ii) a given MED, compared to lighter-skinned subjects.

In this experiment, photoadaptation is studied as a whole entity. Of course, photoadaptation comprises pigmentary and non-pigmentary mechanisms. Pigmentary photoadaptation, by virtue of melanin and melanosome properties, differs according to racial type. Non-pigmentary photoadaptation is gained by epidermal hyperplasia, a process in which biomolecules comprising the epidermis play a role in absorbing UVR, ameliorating its potential damage to DNA. The following chapters will examine how these two components: i) pigmentary, and, ii) non-pigmentary photoadaptation, contribute to the general photoadaptive response as a whole.

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## Chapter 3 \* Pigmentation Study

### 3.1 Introduction

As previously mentioned, photoadaptation encompasses pigimentary and non-pigimentary components. The purpose of this present study is to investigate the pigimentary component in isolation. In doing this, the factors examined will be:

- i) the change in pigimentary colour as an indication of the increase in pigmentation following UVR.
- ii) the time course or temporal pattern of the human pigimentary response, delineating when this response peaks, and when skin pigmentation returns to normal again.
- iii) the dose-response of pigmentation to UVB irradiation.

#### 3.1.1 The Time Course of Pigmentation

The time course or temporal pattern of pigmentation is not well-studied. Therefore, it is of interest to observe how quickly the human skin pigments following UVR exposure, when this response peaks to a maximum, if it plateaus, and when it returns to normal again. Results differ according to the regime and protocol used, depending on whether a single or multiple exposures of UVR are delivered, the type of UVR (UVA/UVB) delivered, and the method used in obtaining measurements of pigimentary change. A study by Choe, et al. of patients undergoing phototherapy showed that following a thrice weekly regime of narrowband UVB, pigmentation increased continuously until the 5<sup>th</sup> week, and then did not progress any further; the study spanned 7 weeks in total<sup>1</sup>. Another study over a four week period found pigmentation to be maximal at Day 7 following a single UVB exposure of 2 MED<sup>2</sup>. An earlier study over a ten week period also found pigmentation to be maximal at Day 7, following exposure to a single UVA and UVB spectrum at 2 MED<sup>3</sup>. A shorter study using visible light of 80J/cm<sup>2</sup> and 120J/cm<sup>2</sup> showed that pigmentation developed immediately following irradiation and decreased significantly after 24 hours, then remained more or less the same through to Day 10<sup>4</sup>. From this, it is evident that studies have utilised different regimes of single or multiple doses of UVR. The effects of a single dose and of multiple doses of irradiation have been studied, with results showing that for a similar total administered dose, a single irradiation is more melanogenic (inducing more melanogenesis and melanocyte activity) than multiple fractionated doses<sup>5</sup>.

#### 3.1.2 Pigimentary Dose-Response

It has been shown that UVB-induced erythema correlates linearly and positively with pigmentation, demonstrating a positive dose-response. In addition, more melanogenesis occurs in dark skin than in light skin for a given level of UV-induced inflammation<sup>6</sup>.

### **3.1.3 Redheads and Non-redheads**

A sub-study of this experiment examined the difference in pigmentary response between two distinct groups of people: Redheads and Non-redheads. Redheads are phenotypically and genotypically distinct, with the key determinant of red hair colour being the Melanocortin-1-Receptor (MC1R) gene. The MC1R gene is located at 16q24.3<sup>7</sup>, coding for a seven-pass-transmembrane receptor, which when activated, ultimately signals to increase the eumelanin:pheomelanin ratio<sup>8</sup>.

Polymorphism of the MC1R gene is related to phenotypic variance in human pigmentation, with a number of MC1R variants existing in the European population<sup>9</sup>. A number of naturally occurring MC1R variants that are associated with red hair display a loss of function of the melanocortin-1-receptor, resulting in its inability to stimulate cAMP production as strongly as the wild type receptor; this then lends to lower eumelanin:pheomelanin ratios, therefore the red hair colour<sup>10</sup>. The alleles frequently associated with red hair colour are R151C, R160W, D294H, 86insA and 537insC, with homozygosity in R151C, R160W and D294H almost always resulting in red hair colour – these are the highly penetrant recessive red hair alleles<sup>11</sup>. In the attempt to understand the relationship between having red hair and pale skin colour (as is commonly the case), Flanagan et al. further found that most individuals with red hair are homozygous or compound heterozygotes for the MC1R variants, whereas non-redheaded individuals with pale skin are more likely to be heterozygotes. Interestingly, it has been shown that there is no difference in the time course of UVB-induced erythema between homozygous and heterozygous MC1R mutants<sup>12</sup>.

Our redheaded subjects were homozygous for loss of function mutations in the MC1R gene, causing a higher ratio of pheomelanin to eumelanin. The rationale for comparing redheads with non-redheads was to prove our expectation of a larger pigmentary increase in non-redheads, given the knowledge that our redheads, by virtue of their genotype, lack the relative capacity to produce eumelanin. The rise in skin melanin content following UVR has been shown to be positively correlated with eumelanin content, but not pheomelanin<sup>13</sup>.

### **3.1.4 Rationale of study design**

The basic gist of this study was for subjects to receive a single exposure of 4 different doses of UVB simultaneously on their lower backs. Following this exposure, the pigmentation colour at the exposed sites was measured and monitored over a 10-12 week period.

#### **3.1.4.1 Time Course of Study**

The time course of this study was over a 10-12 week period, as according to literature<sup>1, 2, 3</sup>, this was thought to be of sufficient duration to capture the rise and fall of pigmentation; moreover, it becomes progressively difficult to maintain the loyalty of subjects beyond the time frame of 12 weeks.



#### **3.1.4.2 UV Source and Exposure Regime**

UVB was used, instead of UVA, as it has greater relevance in the clinical setting of phototherapy today. A single exposure of UVB was used, as this has been shown to be more melanogenic than multiple exposures with fractionated doses<sup>5</sup>.

#### **3.1.4.3 Study Sample**

The group of subjects were purposely chosen to represent a diversity of racial types with various degrees of skin pigmentation. The visible colour of skin is shown to be consistent with the baseline melanin content of skin (constitutive colour); those with darker skin show a greater increase in their melanin content within 1 week post-UV<sup>7</sup>. Constitutive pigmentation is a key determinant and a good predictor of UV sensitivity<sup>14,15</sup>. The relationship between constitutive pigmentation and UV sensitivity has been shown to be dose-dependent<sup>16</sup>. Darker constitutive skin colours lend greater photoprotection against

UVB-induced DNA damage<sup>17</sup>. These people with higher constitutive pigmentation are less likely to burn, and have higher tanning responses following UV<sup>18</sup>. Therefore, the subjects chosen for this study were from diverse racial backgrounds, with the hope that a clear difference can be seen with regards to the degree of pigmentation induced, based on racial background.

The rationale of splitting the subjects into two groups (redheads and non-redheads) further aimed to show the power of the study, with the expectation of demonstrating the ability of non-redheads to develop a greater pigmentary response when compared with redheads, whom we know have a lower eumelanin to pheomelanin production ratio in their pigmentary capacity.

#### **3.1.4.4 Short Study and Long Study**

In the attempt to map the time course of pigmentation, the initial design of the study involved taking pigmentation readings starting at Week 1(or Day 7), and continuing through to Weeks 10-12. As the study got underway, and it became obvious that Week 1/Day7 was an important time-point, it was decided that this early 1<sup>st</sup> week should be examined more closely. Therefore, a separate and shorter study of the

1<sup>st</sup> week was undertaken to map the pigmentary response within the 1<sup>st</sup> week post-UV. Thus, the Short Study detailed the time course within only the 1<sup>st</sup> week post-UV, and the Long Study detailed the time course starting at Week 1(or Day 7) to Week 10-12.

### 3.2 Methods

Altogether, 28 volunteers were recruited from the general Edinburgh population. These 28 volunteers were involved in the two separate studies: 1) the Long Study and 2) the Short Study.

- The **Long Study**, involved 24 subjects: 12 with red hair colour (redheads) and 12 with hair colour other than red (non-redheads). All of the redheads were North European in origin; of the non-redheads however, 2 were Middle Eastern, 2 were Far East Asian, 1 was South Asian (Indian) and 7 were North European. The redheads were selected on the basis of hair colour by visual inspection. Each subject was studied for a period of **9-12 weeks** in the Long Study. The subjects details are on Table 1.
- The **Short Study** involved 6 subjects, 2 of whom were also participants of the Long Study. Each subject was studied over a **1 week** period in the Short Study.

Subjects	Age	Sex	Fitzpatrick Skin Type	Hair	Genotype	Racial origin
Rw	40	M	2	Brown	60 Ht	Nth Eur
Ra	40	M	4	Black	60 Ht	Mid East
Ms	28	F	3	Brown	Wild type	Nth Eur
Cm	28	F	2	Brown	151 Ht	Nth Eur
Jw	60	F	2	Blond	160 Ht	Nth Eur
Jf	66	M	1	Brown	Wild type	Nth Eur
Gf	46	M	2	Brown	160 Ht	Nth Eur
Nb	38	M	4	Black	Wild type	Sth Asian
Kw	40	F	2	Blond	60 Ht 294 Ht	Nth Eur
Th	35	M	3	Black	163 Hm 166 Ht	FE Asian
Tw	28	M	3	Black	92 Ht	FE Asian
Km	33	M	4	Black	Wild type	Mid East
Eh	68	F	1	Red	151 Ht 160 Ht	Nth Eur
As	58	M	2	Red	151 Ht 160 Ht	Nth Eur
Jd	26	F	1	Red	151 Ht 160 Ht	Nth Eur
Ah	41	M	2	Red	151 Ht 294 Ht	Nth Eur
Af	47	M	2	Red	151 Ht 294 Ht	Nth Eur
Ew	42	F	2	Red	151 Ht 294 Ht	Nth Eur
Afn	44	F	1	Red	151 Ht 294 Ht	Nth Eur
Ch	42	F	2	Red	151 Ht 160 Ht	Nth Eur
Ae	24	F	2	Red	151 Ht	Nth Eur
If	42	F	2	Red	151 Ht 160 Ht	Nth Eur
Mn	39	F	2	Red	151 Ht	Nth Eur
Dh	42	M	2	Red	151 Ht 294 Ht	Nth Eur

Table 1. Subject details.

All subjects in both the Long Study and Short Study received 4 set doses of UVB radiation to the right lower back in 4 spots, measuring 1 cm in diameter each. The UVB source was the Xenon Arc Lamp (refer to Chapter 1). The doses delivered were 28mJ/cm<sup>2</sup>, 56mJ/cm<sup>2</sup>, 112mJ/cm<sup>2</sup> and 160mJ/cm<sup>2</sup>; these doses were arranged in a pattern to accommodate 2 unirradiated control spots (Figure 1). The Xenon Arc Lamp was used in this study, as it provided flexibility in delivering any dose that was desired; in addition, the flexible cable enabled placement of the irradiated doses in any desirable arrangement on the skin, as was required in this study.

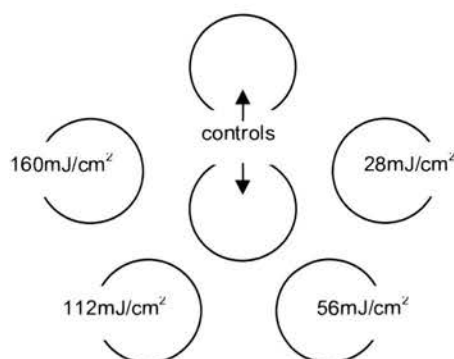


Figure 1. The pattern of irradiated spots on the lower back of a subject.

Over the course of 1 week in the Short Study, the pigmentary colour of these spots was measured on three separate occasions (Day 1, Day 4 and Day 7). Over the course of the 12 weeks in the Long Study, the pigmentary colour of the spots was measured on up to six separate occasions (Week 1, 2, 3, 5, 7-8, 9-12). Pigmentary colour was measured using the Minolta Chromameter and the L\*a\*b\* system. At each time point over the course of the study, triplicate readings were taken of each of the 4 irradiated and 2 control spots and a mean of the readings was then calculated.

In order to obtain measurements of true pigmentary colour, it was essential for UV-induced erythema to first be eliminated from each of the irradiated spots; this was made possible by iontophoresis of noradrenaline through skin as described in Chapter 1. This technique was performed each time pigment colour measurements were taken over the course of the study.

In order to determine if UV-induced erythema was sufficiently suppressed following the iontophoresis process, the Laser Doppler was used to measure skin blood flow readings of each of the 6 spots (refer to Chapter 1). Three consecutive readings were obtained and its mean calculated; this was performed in order to ensure satisfactorily low blood flow prior to pigment colour measurements.

As one group of the study was chosen on basis of red hair colour, the MC1R status of all subjects was determined; this was done by automated sequencing of DNA extracted from whole blood according to previously published methods (Flanagan et al, 2001).

### 3.3 Results

#### 3.3.1 Suppression of Erythema by NA iontophoresis

Erythema was suppressed by the method of NA iontophoresis as described in Chapter 1 prior to the measurement of pigment colour. Doppler flux readings were obtained before and after NA iontophoresis in both Short Study and the Long Study. Figure 2 shows the appearance of pigmentation spots before and after iontophoresis with noradrenaline.

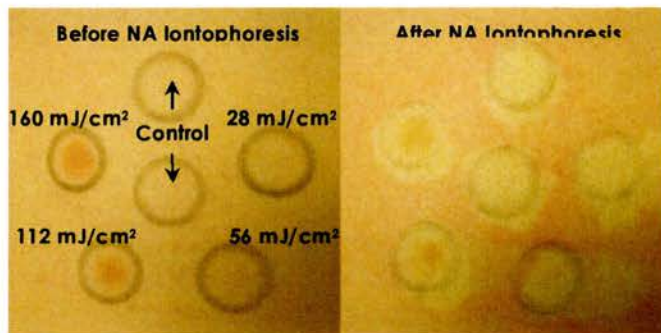


Figure 2. Iontophoresis of noradrenaline onto the irradiated spots results in blanching of the skin of the immediate area, allowing for pigmentation measurements without the influence of erythema.

##### 3.3.1.1 Short Study

In the Short Study, flux was measured before and after NA iontophoresis on Days 1, 4 and 7. Flux measurements were obtained for all four irradiated spots and both unirradiated control spots; a mean of the two unirradiated controls was calculated. The 'increase in flux' was calculated for each of the irradiated spots: (flux of irradiated spot) - (flux of the mean of the unirradiated controls). Tables 1 and 2 detail the flux values before and after NA iontophoresis. There was a lesser increase in flux following NA iontophoresis, than before NA iontophoresis; in other words, the flux of the irradiated spots was more similar to that of the unirradiated controls following iontophoresis with NA. This, of course, was the purpose of NA iontophoresis: to eliminate erythema (measured as flux) in order to obtain true pigmentary colour.

It is worthwhile noting that the flux of normal unirradiated skin on the lower back varies from one individual to another, being as high as 70 flux units. We shall call this 'absolute flux'. Following NA iontophoresis, 'absolute flux' of unirradiated control skin ranged between 17 to 33 flux units. Therefore in this study, all 'absolute flux' values below 40 following NA iontophoresis were acceptable for pigment readings of the irradiated spots. Table 2 details the 'absolute flux' of irradiated and control spots before and after NA iontophoresis.



Mean Absolute Flux before and after NA iontophoresis ( $\pm$ SEM)						
Short Time Course Study						
UVB dose	NA	day 1		day 4		day 7
160mJ/cm <sup>2</sup>	Before	436.16	(85.89)	128.00	(52.90)	69.06 (12.27)
	After	85.13	(14.22)	33.84	(10.96)	26.06 (6.08)
		p=0.01		p=0.13		p=0.04
112mJ/cm <sup>2</sup>	Before	350.39	(93.94)	110.61	(51.24)	50.44 (7.64)
	After	70.60	(28.28)	30.89	(8.87)	25.72 (6.59)
		p=0.04		p=0.19		p=0.07
56mJ/cm <sup>2</sup>	Before	131.95	(32.30)	46.33	(10.33)	31.78 (5.28)
	After	31.33	(2.14)	20.45	(4.18)	24.95 (3.47)
		p=0.01		p=0.07		p=0.36
28mJ/cm <sup>2</sup>	Before	48.78	(5.16)	26.67	(8.23)	31.95 (5.28)
	After	30.93	(3.31)	18.89	(4.32)	22.50 (4.95)
		p=0.02		p=0.16		p=0.24
Control	Before	51.42	(4.14)	25.69	(6.48)	30.14 (3.67)
	After	32.17	(3.28)	17.19	(5.45)	22.95 (6.23)
		p=0.001		p=0.01		p=0.30

Table 2.

The greatest increase in flux occurred at Day 1 post-UVB. It was also apparent that the higher the dose of UVB delivered, the greater was the mean increase in flux above baseline control skin. This dose-response is shown in Figure 3 and Table 3. For example, on Day 1, from the smallest to the greatest UVB dose, the mean increase in flux prior to NA iontophoresis was 0(sem 4.32), 80.53(sem 28.39), 298.97(sem 90.51) and 384.75(sem 82.24). Following NA iontophoresis, the mean increase in flux for the smallest to greatest UVB doses were: 0(sem 1.86), 0(sem 1.96), 38.54(sem 25.58) and 52.97(sem 12.86). Therefore, NA iontophoresis was successful in suppressing the flux of the irradiated spots, as shown in Figure 3.

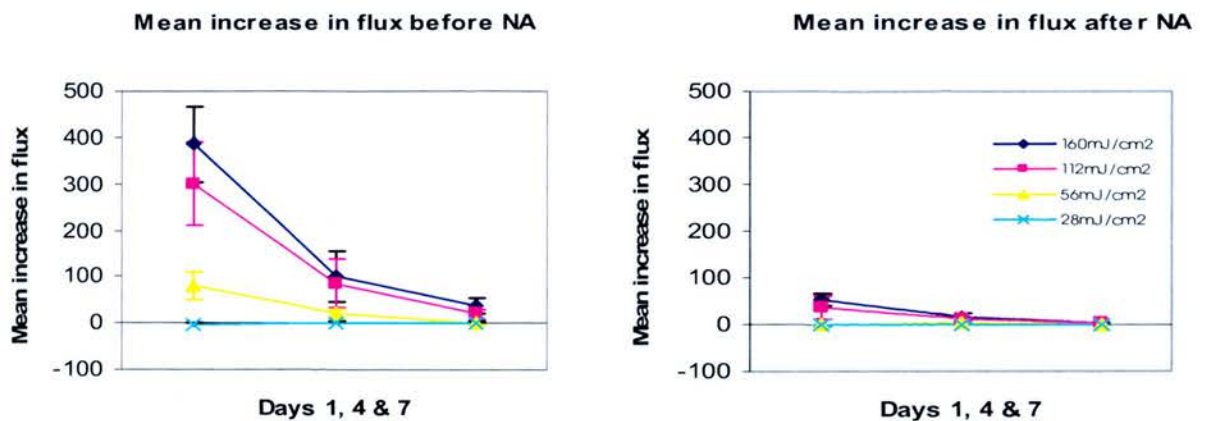


Figure 3. Iontophoresis of noradrenaline decreases flux levels on Days 1, 4 & 7 in the Short Study.

Mean Increase in Flux before and after NA iontophoresis ( $\pm$ SEM)							
Short Time Course Study							
UVB dose	NA	day 1		day 4		day 7	
160mJ/cm <sup>2</sup>	before	384.75	(82.24)	102.31	(54.62)	38.92	(15.28)
	after	52.97	(12.86)	16.64	(6.36)	3.11	(1.26)
		p=0.02		p=0.17		p=0.06	
112mJ/cm <sup>2</sup>	before	298.97	(90.51)	84.92	(52.56)	20.31	(10.45)
	after	38.54	(25.58)	13.69	(4.39)	2.78	(1.30)
		p=0.05		p=0.24		p=0.14	
56mJ/cm <sup>2</sup>	before	80.53	(28.39)	20.64	(10.41)	1.64	(3.04)
	after	-0.84	(1.96)	3.25	(2.54)	2	(4.04)
		p=0.03		p=0.19		p=0.95	
28mJ/cm <sup>2</sup>	before	-2.64	(4.32)	0.97	(2.19)	1.81	(2.96)
	after	-1.23	(1.86)	1.7	(2.56)	-0.44	(1.57)
		p=0.82		p=0.87		p=0.49	

Table 3.

Despite the fact that NA iontophoresis managed to suppress blood flux readings, it only managed to bring the ‘absolute flux’ of 112 mJ/cm<sup>2</sup> and 160mJ/cm<sup>2</sup> down to a mean of 70.20(sem 28.28) and 85.13(sem 14.22) respectively on Day 1 (Table 2). Unfortunately, this was not close enough to the value of normal unirradiated control skin following NA iontophoresis. Therefore, on Day 1 post-UVB, the amount of erythema was too great to suppress, and pigment colour readings on Day 1 may not reflect true pigment colour. However, on Day 4 and Day 7, the mean ‘absolute flux’ of all the spots following NA iontophoresis ranged between 17.19(sem 5.45) and 33.84(sem 10.96), therefore, within the acceptable range for pigment colour readings.

### 3.3.1.2 Long Study

Flux was measured at several time points in the Long Study: Week 1, 2, 3, 5, 7-8 and 9-12. As in the Short Study, flux was measured at each time point before and after NA iontophoresis. Following NA iontophoresis, the absolute flux of all irradiated spots decreased to within an acceptable range of 26 to 34, enabling pigment readings for all irradiated spots over the entire time course (Table 4).

Mean Absolute Flux after NA iontophoresis (± SEM)												
Long Study												
UVB dose	week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	28.67	(2.13)	28.65	(2.07)	34.14	(4.36)	31.15	(1.49)	32.16	(2.42)	28.91	(2.22)
112mJ/cm <sup>2</sup>	32.72	(2.87)	29.35	(2.53)	32.18	(4.28)	30.15	(2.18)	30.96	(1.97)	29.14	(2.41)
56mJ/cm <sup>2</sup>	28.86	(1.97)	29.78	(2.68)	31.16	(3.00)	31.87	(2.16)	30.89	(2.52)	28.98	(2.12)
28mJ/cm <sup>2</sup>	28.84	(2.09)	28.06	(2.21)	31.92	(3.40)	31.33	(2.53)	30.37	(2.19)	29.33	(2.39)
Control	27.54	(1.99)	26.9	(2.08)	30.67	(2.63)	29.67	(1.95)	30.22	(1.76)	28.91	(1.75)

Table 4

Once again, a dose-response was evident; the higher the UVB dose, the greater the flux. Prior to NA iontophoresis on Week 1, the mean increase in flux for UVB doses (28, 56, 112 and 160)mJ/cm<sup>2</sup> were respectively, 0(sem 2.33), 0(sem 3.01), 35.50(sem 9.01) and 84.60(sem 18.93) (see Figure 4, 5 & Table 5)

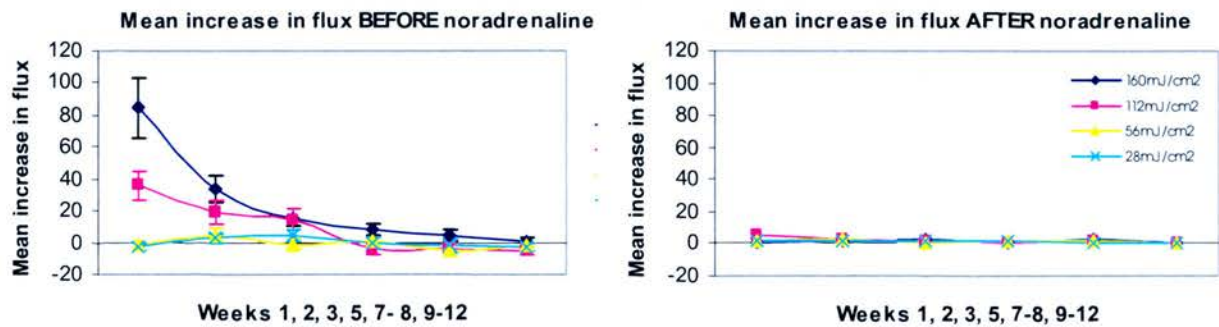


Figure 4. Iontophoresis of noradrenaline reduces flux at time points of Week 1 to Week 12. The dose response is observed by comparing the slopes of the different irradiating doses. The steepest slope with the steepest gradient is seen for the highest dose of 180mJ/cm<sup>2</sup> (see Figure 5), with the lower doses having lesser gradients.

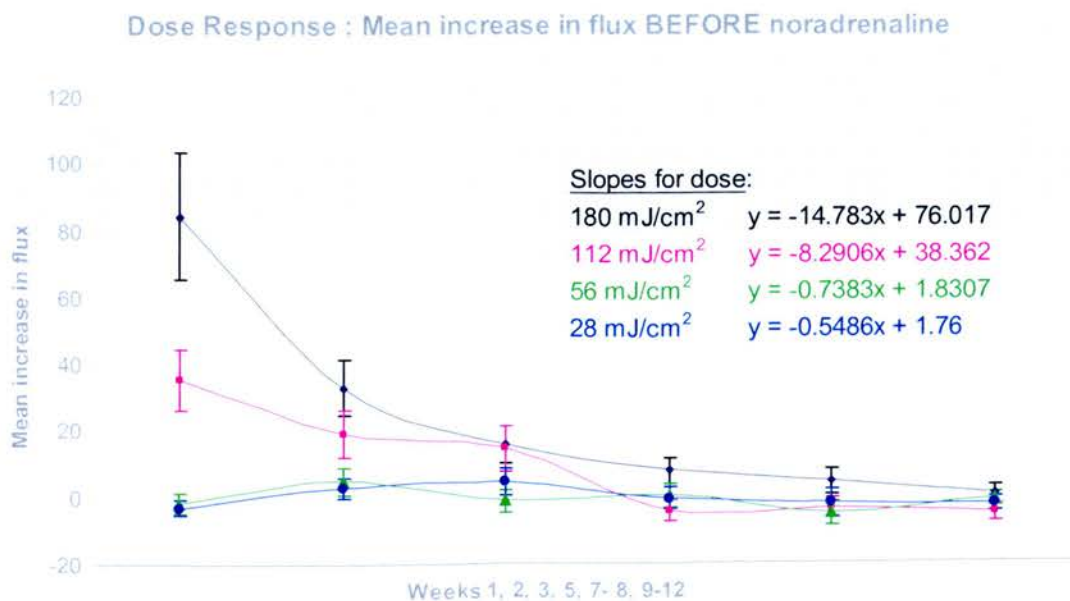


Figure 5. The slopes of the higher doses have progressively higher gradients. The equation for each slope is shown.



Mean increase in flux before and after NA iontophoresis (± SEM)													
Long Study													
UVB dose	NA	week 1		week 2		week 3		Week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	before	84.60	(18.93)	33.19	(8.31)	15.8	(5.66)	7.75	(3.83)	4.19	(4.02)	0.13	(3.02)
	after	1.12	(1.84)	1.75	(0.92)	3.47	(2.89)	0.89	(1.80)	3.24	(1.84)	0.21	(1.71)
		p=0.0003		p=0.002		p=0.08		p=0.16		p=0.82		p=0.98	
112mJ/cm <sup>2</sup>	before	35.50	(9.01)	19.13	(7.18)	14.45	(6.86)	-4.25	(3.27)	-3.61	(3.09)	-5.15	(2.51)
	after	5.17	(2.02)	2.46	(1.15)	1.51	(2.38)	0.04	(1.28)	1.26	(1.36)	0.44	(1.55)
		p=0.003		p=0.03		p=0.08		p=0.23		p=0.09		p=0.08	
56mJ/cm <sup>2</sup>	before	-1.87	(3.01)	4.9	(4.03)	-1.31	(3.41)	0.32	(3.38)	-5.34	(3.62)	-1.22	(2.15)
	after	1.70	(1.14)	2.89	(2.28)	0.49	(1.57)	2.18	(1.52)	1.5	(1.67)	0.28	(1.28)
		p=0.25		p=0.59		p=0.54		p=0.63		p=0.03		p=0.58	
28mJ/cm <sup>2</sup>	before	-3.04	(2.33)	2.98	(2.92)	4.55	(3.96)	-0.49	(3.27)	-2.19	(3.89)	-2.77	(2.04)
	after	1.30	(1.66)	1.75	(0.99)	1.25	(1.71)	1.37	(1.47)	1	(1.37)	1	(1.70)
		p=0.16		p=0.82		p=0.43		p=0.63		p=0.35		p=0.095	

Table 5.

Over the 12 week period, the greatest mean increase in flux (prior to NA iontophoresis) was at Week 1. This was more evident for doses 112mJ/cm<sup>2</sup> and 160mJ/cm<sup>2</sup>. However, following NA iontophoresis, the mean increase in flux of the irradiated spots was very similar to the flux of unirradiated spots, ranging between 0.21(sem 1.71) and 5.17(sem 2.02) flux units above normal unirradiated skin.

### 3.3.2 Measurement of Pigmentation

Pigmentation was measured using the Minolta Chromameter on the L\* scale of L\*a\*b\* (refer Chapter 1). As explained above, pigmentation of the irradiated and unirradiated spots were measured after iontophoresis of NA into the spots. Any increase in pigmentation was calculated as:

$$(L^* \text{ post irradiation}) - (L^* \text{ of control spots ie. constitutive pigmentation}).$$

This method of measurement was chosen for the reason that subjects differed from one another in their baseline/constitutive pigmentation, therefore any change in pigmentation had to be compared to constitutive pigmentation. Changes in pigmentation were not calculated as a percentage of the baseline constitutive value, as these constitutive L\* values were numerically arbitrary, therefore a percentage calculation would not have made sense.

#### 3.3.2.1 Short Study

In the Short Study, pigmentation was measured on Days 1, 4 and 7. For all UVB doses, pigmentation measured by L\* was greatest on Day 7 (Figure 6). For example, following the highest dose of 160mJ/cm<sup>2</sup>, L\* on Days 1, 4 and 7 were respectively, 6.32(sem 1.66), 4.79(sem 0.48) and 6.61(sem 1.08). It is important to note that L\* on Day 1 may not be a valid representation of true pigment colour, as mentioned previously, because erythema was not sufficiently suppressed on Day 1 (see Table 6).



A dose-response was also evident. The mean increase in  $L^*$  above control unirradiated skin for (28, 56, 112 and 160)  $\text{mJ}/\text{cm}^2$  on Day 7 were -0.46(sem 0.51), 1.73(sem 0.89), 4.96(sem 0.94) and 6.61(sem 1.08). Figure 6 and Table 6 also show the dose-response to UVB.

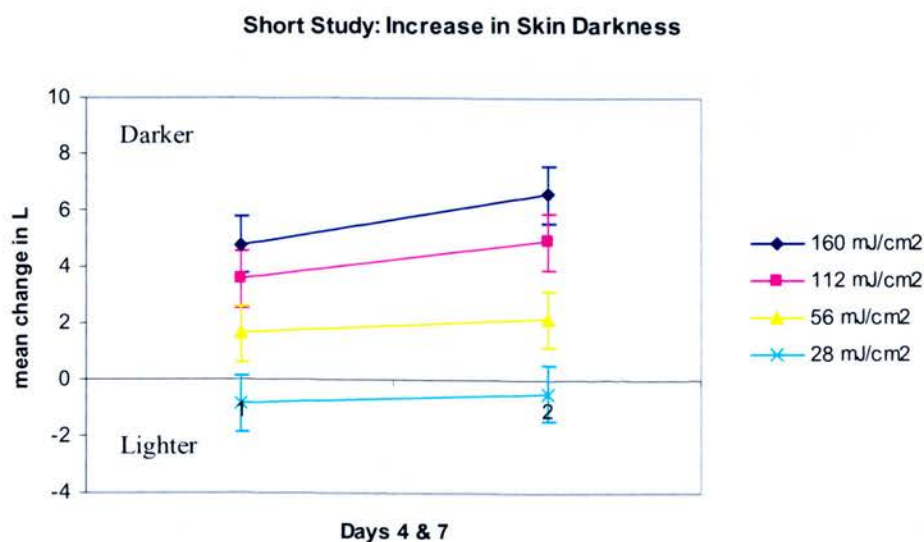


Figure 6. Increase in pigmentation, measured as  $L^*$ , from Day 4 to Day 7, peaking at Day 7 for all irradiated doses.

$L^*$	Day 1		Day 4		Day 7,8	
160 $\text{mJ}/\text{cm}^2$	6.32	(1.66)	4.79	(0.48)	6.61	(1.08)
112 $\text{mJ}/\text{cm}^2$	4.71	(1.64)	3.57	(0.61)	4.95	(0.94)
56 $\text{mJ}/\text{cm}^2$	1.31	(2.18)	1.64	(1.50)	2.18	(0.57)
28 $\text{mJ}/\text{cm}^2$	-0.11	(0.68)	-0.84	(0.42)	-0.46	(0.51)

Table 6. Short Study: Mean increase in  $L^*$  ( $\pm$  SEM)

### 3.3.2.2 Long Study

In the Long Study, pigmentation was measured at Weeks 1, 2, 3, 5, 7-8 and 9-12. It was found that  $L^*$  was highest at Week 1, then decreased at Week 2 and subsequently, continued to decrease (see Figure 7 & Table 7).

Once again, a dose-response was evident, with the highest dose producing higher measures of  $L^*$ : at Week 1, (28, 56, 112 and 160)  $\text{mJ}/\text{cm}^2$  produced a mean increase in  $L^*$  of respectively: 0.62(sem 0.18), 2.55(sem 0.44), 6.19(0.71) and 7.99(sem 0.82). This dose-response pattern was still evident at Weeks 9-12, with the same order of doses producing a mean increase in  $L^*$  of 0.03(sem 0.24), 1.07(sem 0.40), 1.7(sem 0.44) and 1.92(sem 0.44).

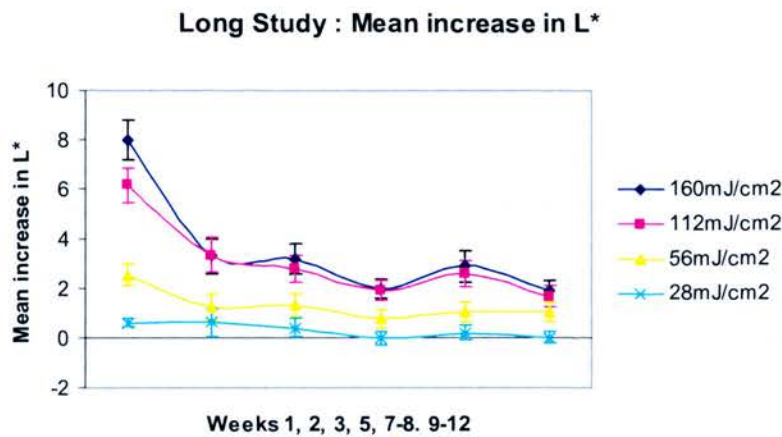


Figure 7. There is a decrease in pigmentation from Week 1 onwards. This graph demonstrates a dose-response to the four different doses of UVB over the 12 week period.

Mean increase in L* (± SEM)												
Long Study												
UVB Dose	week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	7.99	(0.82)	3.31	(0.68)	3.19	(0.58)	2	(0.43)	2.92	(0.63)	1.92	(0.44)
112mJ/cm <sup>2</sup>	6.19	(0.71)	3.35	(0.69)	2.81	(0.51)	1.95	(0.41)	2.63	(0.53)	1.7	(0.44)
56mJ/cm <sup>2</sup>	2.55	(0.44)	1.25	(0.54)	1.34	(0.45)	0.77	(0.35)	1.07	(0.42)	1.07	(0.40)
28mJ/cm <sup>2</sup>	0.62	(0.18)	0.64	(0.55)	0.43	(0.34)	0	(0.24)	0.23	(0.28)	0.03	(0.24)

Table 7

### 3.3.2.3 Redheads vs. Non-redheads

In the Long Study, the pigimentary response of redheads was compared with those of non-redheads.

The genotype of the 12 redheads and 12 non-redheads was determined. Genotyping of the red hair group showed 10 of 12 subjects to be homozygous for known diminished function mutations of the MC1R (Table 8). In the non-redhead group, no subject was homozygous, but several Europeans were heterozygous for diminished and highly penetrant MC1R diversity<sup>9,11,19,20</sup>.

It was found that for each dose, and at each time point, the L\* of non-redheads was lower (darker) than those of redheads (Figure 8). For example, at the highest dose of 160mJ/cm<sup>2</sup> at Week 1, the mean increase in L\* for non-redheads was 9.78 (sem 1.17), whereas that of the redheads was only 6.04 (sem 0.93). (p=0.03). By the end of the study at Weeks 9-12, this difference between redheads and non-redheads was still evident: the mean increase in L\* of non-reds and redheads were respectively, 2.87 (sem 0.82) and 1.21 (sem 0.29) (p=0.06) (Figure 9 & Table 9). The time course of change in L\* appeared to be similar in both groups. Pigmentation however, persisted for a greater length of time in non-redheads compared with redheads.

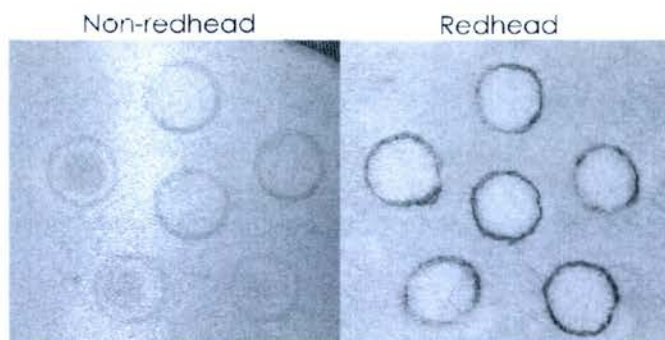


Figure 8. Pigmentation colour after iontophoresis of a non-redhead and a redhead subject taken at 9 weeks post-UVB. Pigmentation is no longer obvious on the redhead, whereas still remains on the non-redhead.

Redheads (n=12)		Non-redheads (n=12)	
Northern European	151 ht 160 ht	Northern European	151 ht
Northern European	151 ht 160ht	Northern European	160 ht
Northern European	151 ht 160ht	Northern European	160 ht
Northern European	151 ht 160ht	Northern European	60 ht 294 ht
Northern European	151 ht 160ht	Northern European	61 ht
Northern European	151 ht 294 ht	Northern European	consensus sequence
Northern European	151 ht 294 ht	Northern European	consensus sequence
Northern European	151 ht 294 ht	Southern Asian	consensus sequence
Northern European	151 ht 294 ht	Middle Eastern	consensus sequence
Northern European	151 ht 294 ht	Middle Eastern	60 ht
Northern European	151 hm	Far East Asian	92 ht
Northern European	160 hm	Far East Asian	163 hm 166 ht

Table 8. Genotype of redheads and non-redheads.



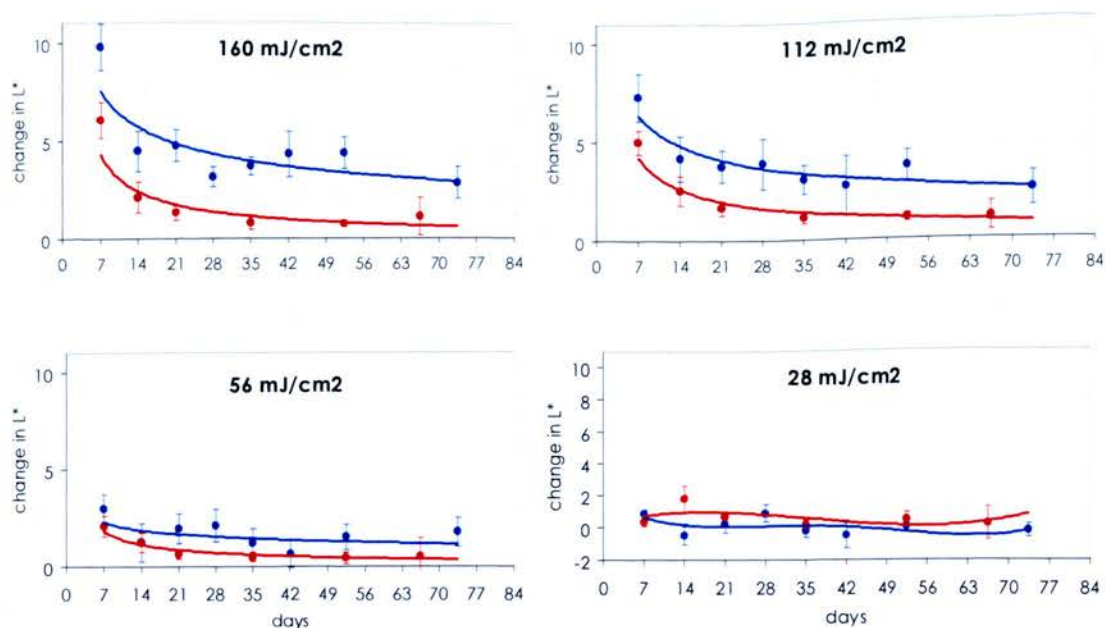


Figure 9. Non-redheads (blue line) pigment to a greater extent, compared to redheads (red line) following the four different UVR doses from Week 1 to Week 12.

Mean increase in L* of Non-redheads and Redheads (± SEM)													
UVB Dose		week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	Non-reds	9.78	(1.17)	4.49	(1.03)	4.74	(0.79)	3.68	(0.46)	4.37	(0.81)	2.87	(0.82)
	Reds	6.04	(0.93)	2.12	(0.79)	1.33	(0.37)	0.78	(0.32)	0.75	(0.17)	1.21	(0.29)
		p=0.03		p=0.04		p=0.01		p=0.01		p=0.01		P=0.01	
112mJ/cm <sup>2</sup>	Non-reds	7.29	(1.21)	4.17	(1.13)	3.77	(0.81)	3.06	(0.72)	3.66	(0.75)	2.44	(0.87)
	Reds	4.98	(0.62)	2.54	(0.76)	1.66	(0.36)	1.14	(0.32)	1.08	(0.19)	1.13	(0.22)
		p=0.23		p=0.10		p=0.11		p=0.14		p=0.03		P=0.16	
56mJ/cm <sup>2</sup>	Non-reds	3	(0.70)	1.26	(0.99)	1.92	(0.76)	1.17	(0.77)	1.5	(0.64)	1.76	(0.76)
	Reds	2.07	(0.56)	1.24	(0.48)	0.64	(0.26)	0.48	(0.23)	0.43	(0.31)	0.49	(0.31)
		p=0.55		p=0.57		p=0.33		p=0.48		p=0.51		p=0.16	
28mJ/cm <sup>2</sup>	Non-reds	0.88	(0.27)	-0.52	(0.57)	0.21	(0.56)	-0.21	(0.39)	0.05	(0.36)	-0.2	(0.39)
	Reds	0.34	(0.24)	1.79	(0.83)	0.69	(0.35)	0.15	(0.31)	0.49	(0.46)	0.24	(0.32)
		p=0.21		p=0.06		p=0.36		p=0.20		p=0.40		p=0.21	

Table 9

Apart from examining pigmentation with regard to hair colour and its implications, pigmentation was also examined from the point of view of the amount of erythema generated as a result of UV irradiation.

It was of interest to see if there was any relationship between the amount of UV-induced erythema and the resulting pigmentation response. For example, does more erythema result in darker pigmentation?

Or conversely, does more erythema result in less pigmentation, under the assumption that those who suffer greater UV-induced erythema are usually of skin phototypes 1 or 2 and therefore, may not pigment as easily as those of darker phototypes. Was there a relationship between erythema and pigmentation?

Figure 10, below, shows that at Week 1, firstly the non-redheads were grouped towards the left, with very little erythema. Secondly, there was no relationship between erythema and pigmentation at that point in time. It is regrettable that the subject number was small, with only 7 non-redheads and 12 redheads. This result is in contradiction to results of a recent large study of 98 subjects, showing that erythema is a predictor of pigmentation (results yet to be published; personal communication: Professor Jonathan L Rees) The researchers state that their finding on pigmentation being proportional to erythema is true only if all other factors are kept constant.

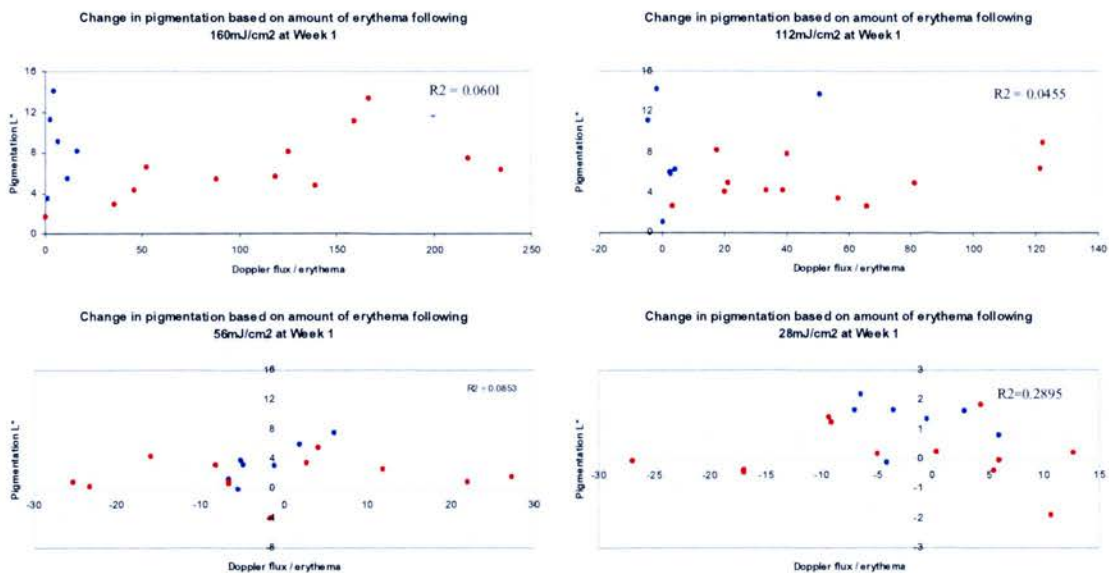


Figure 10. The change in pigmentation examined according to the amount of UV-induced erythema. There is no correlation between the amount erythema produced and the amount of pigmentation generated as a result of the UV insult. Blue dots represent non-redheads, red dots represent redheads. The  $R^2$  value is given in each graph for a trendline that would have been drawn through the plot.

Therefore, to answer the question on whether, for a certain amount of erythema, there was a pattern of distribution for pigmentation in redheads and non-redheads in our study, unfortunately, the range of erythema experienced by our subjects was too wide (especially for the higher doses of 112 mJ/cm<sup>2</sup> and 160 mJ/cm<sup>2</sup>) to allow for comparison of pigmentation induced from a small given range of erythema. Nevertheless, with the information from Figure 10 above, it can be seen within this present small study that throughout the range of erythema on the x-axis, the scatter of pigmentation responses is random. A larger study is needed in order to investigate the relationship and predictive value between erythema and pigmentation.

### 3.3.3 Colour Measurement by a\*

#### 3.3.3.1 Short Study

Colour was measured on the a\* scale on Days 1, 4 and 7 in the Short Study. Figure 11 and Table 10 show values of a\* on these occasions. A dose-response was observed, with higher doses of UVB producing higher readings on the a\* scale; this held true over the course of the week. For the various doses from Day 4 to Day 7 the a\* readings were as follows: for 160mJ/cm<sup>2</sup>: 3.02(sem 0.31) to 3.6(sem 0.27)(p=0.16); for 112mJ/cm<sup>2</sup>: 2.22(sem 0.47) to 2.3(sem 0.74)(p=0.85); for 56mJ/cm<sup>2</sup>: 0.2(sem 0.53) to 1.56(sem 0.35)(p=0.06); for 28mJ/cm<sup>2</sup>: -0.4(sem 0.36) to 0.02(sem 0.24)(p=0.51).

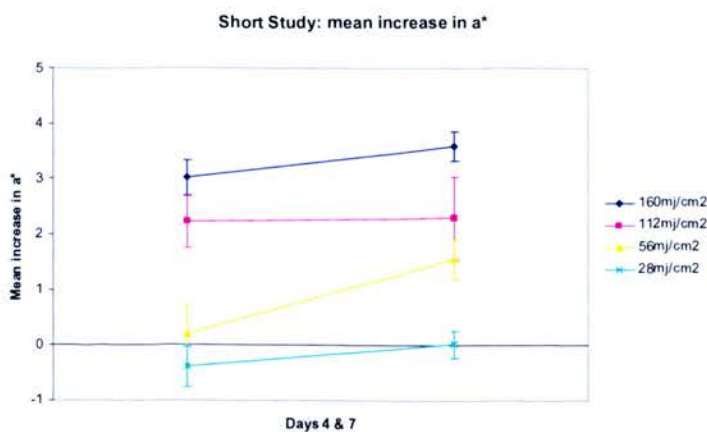


Figure 11. Increase in a\* from Day 4 to Day 7

a*	Day 1	Day 4	Day 7,8
160mJ/cm <sup>2</sup>	6.99 (2.43)	3.02 (0.31)	3.6 (0.27)
112mJ/cm <sup>2</sup>	4.82 (2.46)	2.22 (0.47)	2.3 (0.74)
56mJ/cm <sup>2</sup>	1.96 (1.52)	0.2 (0.53)	1.56 (0.35)
28mJ/cm <sup>2</sup>	0.58 (0.61)	-0.4 (0.36)	0.02 (0.24)

Table 10: Short Study: Mean increase in a\* (± SEM)

#### 3.3.3.2 Long Study

Colour measurements were taken on Weeks 1, 2, 3, 5, 7-8 and 9-12. It was shown that the highest a\* readings were obtained at Week 1 for all doses (Figure 12 & Table 11). The a\* readings of Week 1 vs. Week 2 showed that a\* was highest at Week 1 and subsequently fell at Week 2.

There was also a dose-response; for example, at Week 1, a\* readings from the smallest to largest UVB doses were 0.05 (sem 0.19), 1.51 (sem 0.30), 4.3 (sem 0.55) and 5.79 (sem 0.74).



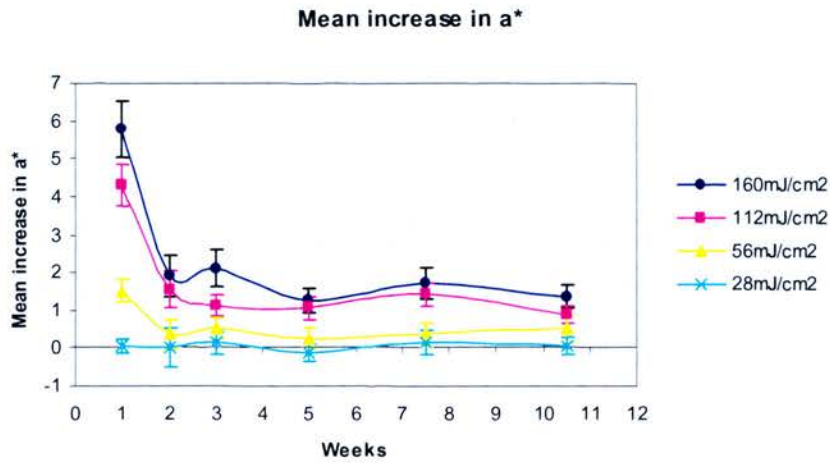


Figure 12. The change in a\* from Week 1 to Week 12 for all four doses of UVB.

Mean increase in a* (± SEM)												
Long Study												
UVB Dose	week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	5.79	(0.74)	1.9	(0.55)	2.12	(0.48)	1.25	(0.33)	1.71	(0.42)	1.36	(0.30)
112mJ/cm <sup>2</sup>	4.30	(0.55)	1.56	(0.50)	1.12	(0.28)	1.06	(0.31)	1.44	(0.31)	0.89	(0.22)
56mJ/cm <sup>2</sup>	1.51	(0.30)	0.37	(0.39)	0.53	(0.27)	0.23	(0.28)	0.37	(0.29)	0.52	(0.37)
28mJ/cm <sup>2</sup>	0.05	(0.19)	0.01	(0.52)	0.15	(0.30)	-0.12	(0.24)	0.14	(0.32)	0.06	(0.24)

Table 11

The mean increase in a\* of non-redheads was compared with that of redheads. In both groups, there was a dose response; for the redheads, this became less evident with time (Figure 13 & Table 12)

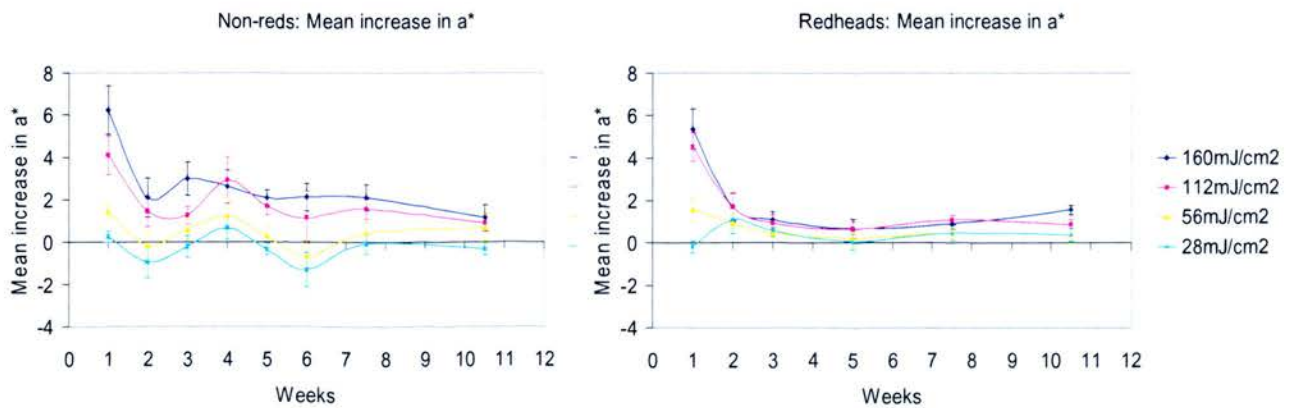


Figure 13. Comparing the mean increase in a\* of redheads and non-redheads

Mean increase in a* of Non-redheads and Redheads ( $\pm$ SEM)											
UVB Dose		week 1		week 2		week 3		week 5		week 7-8	
160mJ/cm <sup>2</sup>	Non-reds	6.24	(1.15)	2.11	(0.92)	2.99	(0.76)	2.06	(0.37)	2.11	(0.64)
	Reds	5.34	(0.96)	1.69	(0.64)	1.07	(0.36)	0.66	(0.44)	0.88	(0.21)
		p=0.55		p=0.42		p=0.07		p=0.07		p=0.37	
112mJ/cm <sup>2</sup>	Non-reds	4.08	(0.91)	1.43	(0.73)	1.27	(0.41)	1.67	(0.42)	1.56	(0.47)
	Reds	4.52	(0.67)	1.68	(0.70)	0.93	(0.37)	0.62	(0.40)	1.07	(0.22)
		p=0.89		p=0.71		p=0.96		p=0.19		p=0.79	
56mJ/cm <sup>2</sup>	Non-reds	1.45	(0.28)	-0.17	(0.62)	0.60	(0.46)	0.27	(0.50)	0.43	(0.51)
	Reds	1.57	(0.55)	0.90	(0.43)	0.44	(0.22)	0.19	(0.33)	0.46	(0.26)
		p=0.89		p=0.35		p=0.96		p=0.98		p=0.88	
28mJ/cm <sup>2</sup>	Non-reds	0.28	(0.26)	-0.96	(0.71)	-0.21	(0.50)	-0.34	(0.28)	-0.07	(0.48)
	Reds	-0.19	(0.26)	1.07	(0.66)	0.58	(0.27)	0.04	(0.37)	0.45	(0.36)
		p=0.04		p=0.08		p=0.40		p=0.17		p=0.07	

Table 12

### 3.3.4 Colour Measurement by b\*

#### 3.3.4.1 Short Study

Colour was measured on the b\* scale on Days 1, 4 and 7. Figure 14 and Table 13 show the b\* values obtained on the relevant occasions. There was no clear dose-response demonstrated on the b\* scale.

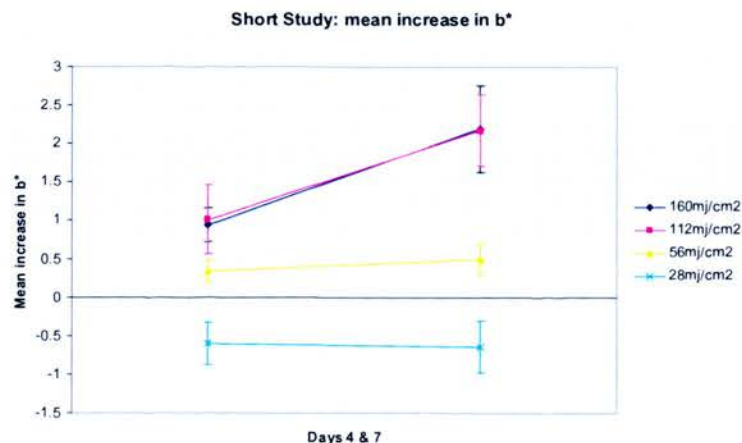


Figure 14. Values on the b\* scale did not demonstrate a clear dose-response.

b*	Day 1		Day 4		Day 7,8	
160mJ/cm <sup>2</sup>	0.29	(0.30)	0.94	(0.22)	2.20	(0.56)
112mJ/cm <sup>2</sup>	0.71	(0.66)	1.01	(0.45)	2.18	(0.46)
56mJ/cm <sup>2</sup>	0.01	(0.37)	0.34	(0.14)	0.49	(0.20)
28mJ/cm <sup>2</sup>	-0.59	(0.31)	-0.60	(0.27)	-0.64	(0.34)

Table 13. Short Study: Mean increase in b\* ( $\pm$  SEM)



### 3.3.4.2 Long Study

Colour was measured on the b\* scale at Weeks 1, 2, 3, 5, 7-8 and 9-12 (Figure 15 & Table 14). A dose-response was not demonstrated by the b\* values over the course of the Long Study.

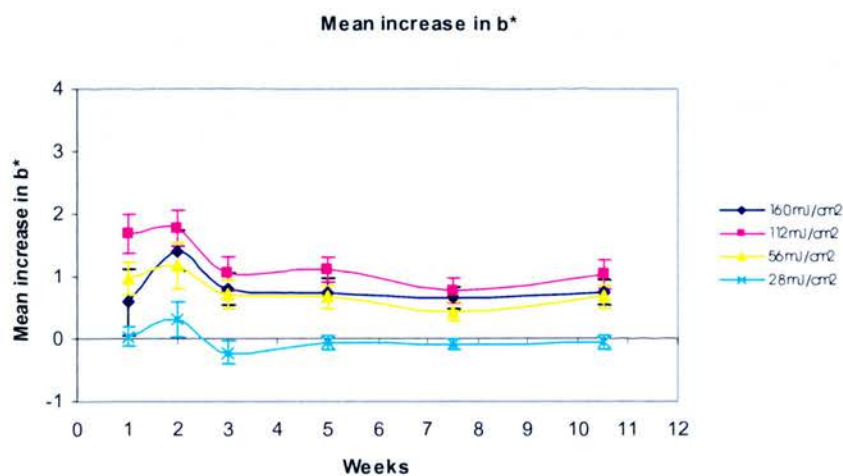


Figure 15. Values on the b\* scale did not demonstrate any dose-response.

Mean increase in b* (± SEM)												
Long Study												
UVB Dose	week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	0.59	(0.52)	1.41	(0.33)	0.8	(0.27)	0.74	(0.24)	0.67	(0.17)	0.74	(0.19)
112mJ/cm <sup>2</sup>	1.68	(0.31)	1.78	(0.29)	1.05	(0.27)	1.12	(0.20)	0.76	(0.20)	1.04	(0.23)
56mJ/cm <sup>2</sup>	0.97	(0.25)	1.17	(0.37)	0.72	(0.22)	0.68	(0.18)	0.44	(0.16)	0.68	(0.18)
28mJ/cm <sup>2</sup>	0.04	(0.15)	0.31	(0.28)	-0.22	(0.18)	-0.06	(0.12)	-0.08	(0.09)	-0.05	(0.12)

Table 14

The mean increase in  $b^*$  of non-redheads was also compared with that of redheads. There was no evidence of a dose response in either group, nor was there a consistent pattern between the two groups (Figure 16 & Table 15).

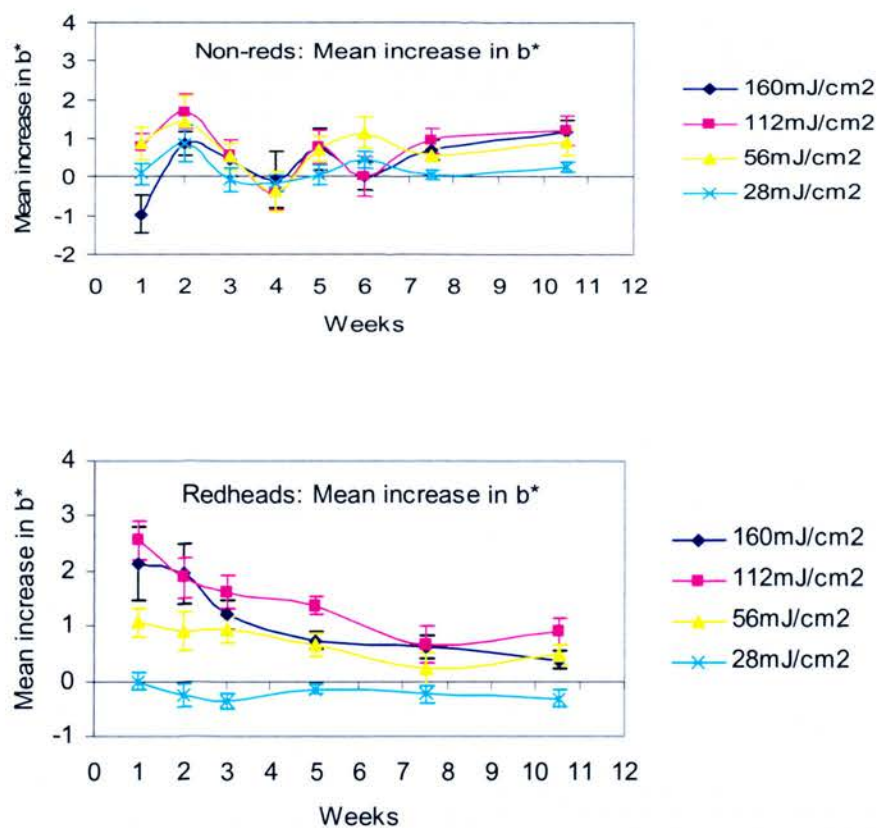


Figure 16. Mean increase in  $b^*$  of Non-redheads and Redhead does not demonstrate a dose-response.

Mean increase in $b^*$ of Non-redheads and Redheads ( $\pm$ SEM)													
UVB Dose		week 1		week 2		week 3		week 5		week 7-8		week 9-12	
160mJ/cm <sup>2</sup>	Non-reds	-0.95	(0.49)	0.87	(0.31)	0.45	(0.44)	0.73	(0.54)	0.70	(0.26)	1.16	(0.31)
	Reds	2.12	(0.67)	1.95	(0.55)	1.21	(0.26)	0.74	(0.17)	0.62	(0.22)	0.39	(0.16)
112mJ/cm <sup>2</sup>	Non-reds	0.80	(0.35)	1.69	(0.45)	0.58	(0.39)	0.79	(0.42)	0.94	(0.31)	1.21	(0.40)
	Reds	2.56	(0.36)	1.88	(0.37)	1.62	(0.29)	1.37	(0.15)	0.67	(0.32)	0.90	(0.25)
56mJ/cm <sup>2</sup>	Non-reds	0.88	(0.42)	1.43	(0.67)	0.53	(0.36)	0.71	(0.32)	0.58	(0.17)	0.91	(0.34)
	Reds	1.06	(0.27)	0.90	(0.36)	0.94	(0.24)	0.65	(0.22)	0.22	(0.30)	0.48	(0.16)
28mJ/cm <sup>2</sup>	Non-reds	0.09	(0.27)	0.87	(0.47)	-0.09	(0.30)	0.05	(0.27)	0.05	(0.12)	0.27	(0.13)
	Reds	-0.01	(0.16)	-0.26	(0.22)	-0.36	(0.15)	-0.14	(0.09)	-0.24	(0.15)	-0.32	(0.15)

Table 15

### 3.4 Discussion

The need to measure pigmentation without the influence of erythema or blood flow is important. The overlap in spectra for haemoglobin and melanin means that methods based on reflectance pose problems<sup>21,22</sup>. Either one uses  $a^*$  or  $L^*$  scores, and obtains data that are subject to systematic error, or alternatively, one can rely on linear extrapolation from readings greater than 650nm<sup>18</sup>. The method used in this study employs a more direct approach in eliminating erythema by use of noradrenaline. Doppler flowmetry was used to assess blood flow as a measure of vascular response or erythema<sup>23</sup>. This allowed for colour measurements using the  $L^*a^*b^*$  scale as a non-invasive quantitative measure of pigmentation; it also allowed for serial measures on the same person.

It is shown that iontophoresis with NA can inhibit flux at all but the earliest stages following UVR. Demonstrated is a dose-response, with higher doses of UVR producing greater degrees of erythema measured as flux. Blood flux was highest on Day 1, to the extent that it was insuppressible by NA iontophoresis; this was the only point in time that any measurement of pigmentation was not valid. Unfortunately, this was a flaw in our method. Nevertheless, it was unlikely for pigmentation to have been greater on Day 1 than on Day 4, as the data showed an increase in pigmentation from Day 4 to Day 7, peaking at Day 7 itself. Apart from Day 1, the method of NA iontophoresis was successful in suppressing blood flux or erythema to the extent that the flux of irradiated spots post-NA was similar to the flux of control unirradiated spots post-NA iontophoresis. This means that one can assume that the only difference between irradiated and control unirradiated skin was due to hyperpigmentation. Therefore,  $L^*$  readings that were taken following NA iontophoresis was as true as possible to colour produced by pigmentation only. Using this method, pigment measurements were recorded over the entire 12 week period. This novel method<sup>24</sup> is a strong point in this study and we hope that it will be employed by future investigators.

Examining the entire study, readings of pigmentation taken from Day 4 to Week 12 showed that, following a single dose of UVB, pigmentation was at its darkest on Day 7. Thereafter, it then declined, but was still present above basal levels even after 10-12 weeks. Unfortunately, our study only spanned 12 weeks. In order to determine the time at which facultative pigmentation (pigmentation gained by intervention over one's own basal pigmentation level) declines to the level of one's constitutive pigmentation, future studies would need to follow subjects for longer than 12 weeks. I suspect that the time to reach constitutive pigmentation is different for different skin types – this adds another factor to the issue of the return to constitutive levels – yet another matter for future studies.

A dose-response was demonstrated over the time course, as one would expect, with higher doses of UVB producing darker pigmentation. This is evidenced in both the redheads and non-redheads. These two groups differed in genotype with respect to their MC1R status. As expected, there was a difference between these two groups in their development of facultative pigmentation. Following the same dose

of UVB for both groups, non-redheads developed pigmentation to a greater extent, when compared with redheads. Both groups showed a similar dose-response; the time course of disappearance of pigmentation was also similar in both groups. In other words, their rate of pigment loss was similar, despite the fact that they attained different pigment levels following UV exposure.

Unlike the  $L^*$  value, both the  $a^*$  value and  $b^*$  value did not yield meaningful results. Despite demonstrating a dose-response and being highest within the first week; there was no significant difference in  $a^*$  between Day 4 and Day 7. There was also no significant difference in  $a^*$  between redheads and non-redheads. The  $b^*$  values showed neither a dose-response nor a consistent trend over the time course of the entire study. The  $b^*$  values were not helpful in differentiating redheads from non-redheads.

In conclusion, this study strove to elucidate the behaviour of pigmentary photoadaptation. The time course of pigmentation in the photoadaptive response of human skin was followed over a 12 week period, with results showing a peak in pigmentation on Day 7 following a single exposure to UVB. This peak in pigmentation was consistent in 2 groups of people with differing pigmentary characteristics based on hair colour and genotype. A dose-response was successfully demonstrated over the entire period of the study, showing that pigmentary photoadaptation can be upregulated to match the UV stimulus. The differences and similarities in the pigmentary photoadaptive response of redheads and non-redheads were outlined. Redheads pigmented to a lesser extent following a single set dose of UVB; however, their rate of pigment loss was the same as those of non-redheads. Given that redheads lack the full capacity to photoadapt via pigmentation, it is thought that there has to be a non-pigmentary photoadaptive mechanism which protects this group of people from UV damage. This forms the topic for the following chapter.

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## Chapter 4 \* Epidermal Thickness & Melanin Study

### 4.1 Introduction

The previous chapters looked into photoadaptation as a whole and subsequently, the increase in pigmentation as one component of the photoadaptive response. This current chapter serves to investigate the other component of the photoadaptive response: epidermal thickening. This is done using UV Transmission Spectrophotometry which, in addition, provides information on the melanin density of the epidermis. This measure of melanin density is further confirmed by quantification of melanin by a known chemical assay<sup>1</sup>.

It is known that repeated UVB exposure promotes epidermal hyperplasia in mouse experiments<sup>2,3</sup>. Earlier human experiments found UV radiation to induce changes in the epidermis such as hyperkeratosis, parakeratosis and acanthosis<sup>4</sup>. This, of course, increases the thickness of the epidermis. In this study, we aim to measure any increase in thickness of the epidermis following UV exposure. In attempting to do so, one needs to remove the epidermis from the underlying dermis. Various methods of removing epidermis for purposes of measuring its thickness have been devised<sup>5</sup>. Our method of removing the epidermis is by suction blistering (refer to Chapter 1).

Experimentally, there are various methods of measuring epidermal thickness. In this study, the method employed is Transmission Spectrophotometry. The method of spectrophotometry has been used for the evaluation skin colour and pigmentation since the early twentieth century, as well as for research into carcinogenesis<sup>6,7</sup>. Transmission Spectrophotometry allows us to decipher the absorption spectrum of melanin, which is in the ultraviolet and visible wavelengths<sup>8,9</sup>. Apart from melanin, other biomolecules exist in the epidermis, which also have an absorption profile. The absorption spectrum of human epidermis through the ultraviolet and visible spectra has been studied by observation of the transmission of these wavelengths through epidermal samples<sup>10</sup>. When incident wavelengths travel through skin, absorption and scattering of these wavelengths are influenced by structures and pigment within the skin, giving the skin its final optical appearance and colour<sup>11</sup>.

Transmission studies enable the calculation of absorbance by the epidermis and any scattering that takes place within the epidermis. It is then possible to calculate the thickness of the epidermis using a mathematical model that takes into account the absorbance due to biomolecules, melanin and any scattering within the epidermis<sup>12</sup>. Bruls et al. found a good correlation between epidermal thickness measurements using Transmission Spectrophotometry and microscopy<sup>13</sup>.

UV transmission studies on split skin samples have found increased ultraviolet protection following a prior UVB exposure<sup>14</sup>. It may be logical to think that the increased ultraviolet protection is at least partly attributed to the increase in epidermal thickness. Lock-Andersen et al. found UV sensitivity to be influenced by constitutive pigmentation, but independent of epidermal thickness<sup>15</sup>. Therefore, there



is discrepancy in the literature on the matter of epidermal thickening as a response to UV exposure. This study, therefore, aims to clarify whether epidermal thickening is a response of the human skin following UV exposure.

## **4.2 Methods**

### **4.2.1 Subjects**

20 healthy volunteers were recruited from the Edinburgh area, comprising 16 North Europeans and 4 Asians. All of the North Europeans were from the UK; 3 of the Asians were from South East Asia and 1 from India. 8 volunteers with vitiligo were also recruited for the study.

### **4.2.2 UV irradiation**

Three doses of 'Adaptation Doses' of UVB were delivered to the right lower back of the 20 volunteers (without vitiligo) over three consecutive days; the doses delivered were: 480mJ/cm<sup>2</sup>, 520mJ/cm<sup>2</sup> and 570mJ/cm<sup>2</sup>. The irradiated area was a square of 9cm x 9cm on the right lower back. The UVB source was the Waldmann TL-01 Lamp (refer to Chapter 1). This procedure was performed to promote a photoadaptive response on the right lower back. The adjacent left lower back was left unirradiated, serving as an unadapted control.

Once again, as in the Photoadaptation Study, the Waldman TL-01 Lamp was used for photoadapting because it provided a large irradiating surface. Later, this area was then challenged with the wideband Diffey Lamp (described below in 2.6), as it was convenient for delivering three different doses simultaneously within close proximity of one another.

### **4.2.3 Suction Blister and Spectrophotometry**

The time course of pigmentation was previously determined, with pigmentation found to be maximal between 1 and 2 weeks<sup>16</sup>; therefore 13 days after the final irradiation, suction blisters were formed and blister roofs (full thickness epidermis) were removed from both the unirradiated and irradiated areas of the lower back of the 20 volunteers. Suction blisters were also formed on the vitiliginous patches and adjacent normal skin of the 8 subjects with vitiligo. The blister roofs of 1cm diameter were excised using fine scissors and suspended in physiological-buffered saline in a quartz cell to eliminate dehydration of the sample. The quartz cell is an ideal chamber for small skin specimens prepared for UV transmission studies and has been previously used by Hoffman et al<sup>17</sup>. The quartz cell was then placed in the optical path of the Fluoromax-3 Spectrophotometer (refer to Chapter 1) operated in synchronous scanning mode; its position was at the entrance aperture of an integrating sphere, which collects all forward transmitted and scattered radiation. The spectral transmittance (relative to a cell containing only saline) was obtained over the spectral region 250-400nm in 1nm steps; the spectral absorbance was derived as the logarithm (to base 10) of the reciprocal of spectral transmittance.

#### 4.2.3.1 The Mathematical Model and the Use of Vitiliginous Skin

The mathematical model used in obtaining thickness measurements and melanin density by UV Spectrophotometry is well described<sup>12</sup>:

$$\text{Absorbance}(\lambda) = \text{Absorbance}(\lambda)_{\text{biomolecules}} + \text{Absorbance}(\lambda)_{\text{melanin}} + \text{Scatter}.$$

In the equation above, total optical absorbance by epidermis ( $\text{Absorbance}(\lambda)$ ) is due to i) absorbance by biomolecules ( $\text{Absorbance}(\lambda)_{\text{biomolecules}}$ ) such as amino acids and nucleic acids, ii) absorbance by melanin ( $\text{Absorbance}(\lambda)_{\text{melanin}}$ ), and iii) scattering. It is important to bear in mind that attenuation due to scattering is important to consider when looking at absorbance in the region of melanin absorbance ( $>330\text{nm}$ ), but it is less important when looking at the region of absorbance due to biomolecules ( $<310\text{nm}$ ). This is because scattering accounts for a relatively larger proportion of the absorbance profile in the region of melanin compared to that in the region of biomolecules; in the region  $<310\text{nm}$ , absorbance due to biomolecules is so great that the proportion due to scattering is negligible.

The 8 volunteers with vitiligo were used to firstly determine the absorbance profile that is due to scattering within the epidermis. As melanin is absent in vitiliginous skin, any absorbance is due to only two things: i) biomolecules and ii) scattering. Since the absorbance profile of biomolecules is in the shorter wavelengths ( $<310\text{nm}$ ), any absorbance in the longer wavelengths from  $360\text{-}400\text{nm}$  in vitiliginous skin is due to scattering. The function due to scattering is therefore readily available in vitiliginous skin, and this function can then be extrapolated to the shorter wavelengths ( $<310\text{nm}$ ) where absorbance due to biomolecules predominates. Subtracting the scattering function from the total absorbance of vitiliginous epidermis in the shorter wavelengths, one is left with absorbance due to biomolecules only. This resulting absorbance that is derived translates to thickness, as the amount of biomolecules represents the amount of epidermis there is, or technically, epidermal thickness. Therefore, in the samples of vitiliginous epidermis, we were able to determine absorbance due to scattering and also absorbance due to thickness of the epidermis.

The next step is to determine, in normal unirradiated Caucasian epidermis, the function due to scattering, thickness and melanin. Remember that we had previously worked out scattering and thickness (biomolecule absorbance) in vitiliginous skin. In normal unirradiated Caucasian epidermis, firstly, absorption due to melanin can be approximated by a known Gaussian function peaking at  $310\text{nm}$ <sup>18</sup>. This function can be fitted between  $330\text{nm}$  -  $400\text{nm}$  on a graph of total absorbance; subtracting this fitted curve from total absorbance, one is left with absorbance due predominantly to biomolecules. The next step is to determine the scattering function of this normal epidermis. This is derived by multiplying the scattering function of vitiliginous skin (which we previously worked out as above) with the ratio of 'areas under the curves' of the absorbance profile due to biomolecules for normal Caucasian epidermis and vitiliginous epidermis. Thus is derived the scattering function for normal Caucasian epidermis. Now that this is available, this scattering function is subtracted from the

total absorbance. Once it is subtracted, the spectral region above 330nm represents absorbance due solely to melanin. Likewise, the region < 310 shows absorbance due solely to biomolecules, therefore represents epidermal thickness.

This sequence of steps is carried out for all irradiated and unirradiated samples of epidermis when determining absorbances due to thickness (biomolecules), melanin and scattering. The unirradiated Caucasian epidermis (as worked out above) is taken as the reference sample, and the absorbances of other samples are calculated by comparing individual areas under the curves for biomolecules, melanin and scatter. Thus calculations done in this way take into account changes in the epidermis, its quality, thickness and constituents. As the scattering function is known to change according to the variables mentioned<sup>11</sup>, this is accounted for by this method. Of course, by this method, calculations of epidermal thickness, melanin content and scattering are derived and are not direct measurements. However, it is useful in that a UV Transmission Spectroscopy can provide indirect measurements of both epidermal thickness and melanin content.

#### **4.2.4 Melanin Analysis by Chemical Degradation**

After obtaining the transmission/absorbance information of the blister roofs in the previous step, the blister roofs were freeze-dried and subjected to chemical degradation for quantification of melanin. Chemical degradation of eumelanin by acidic permanganate oxidation formed pyrrole-2,3,5-tricarboxylic acid (PTCA); hydriodic acid reductive hydrolysis of pheomelanin formed 4-amino-3-hydroxyphenylalanine (4-AHP). The concentrations of these degradation products were then determined using HPLC assays, and then converted to eumelanin and pheomelanin concentrations by multiplying by factors of 160 for eumelanin, and 9 for pheomelanin<sup>1</sup>.

#### **4.2.5 Pigment Colour Measurement**

During the harvesting of suction blister roofs from the adapted and unadapted lower back, the pigment colour of both sites was recorded using the Minolta Chromameter which uses the L\*a\*b\* system. It measures the reflectance of the right and left lower back skin over the wavelengths of 360nm to 740nm.

This enabled comparison of the pigment colour between the adapted and unadapted skin.

Noradrenaline iontophoresis was performed prior to pigment colour measurements as described in Chapter 1.

#### **4.2.6 Measurement of Erythema following UVB Challenge**

During the harvesting of suction blister roofs, both the right (adapted) and left (unadapted) lower back of 16 out of the 20 subjects were challenged with a series UVB delivered by the Diffey Machine (refer to Chapter 1). The doses were delivered in small spots of 0.8cm in diameter. The doses delivered were 38mJ/cm<sup>2</sup>, 47mJ/cm<sup>2</sup>, 60mJ/cm<sup>2</sup>, 75mJ/cm<sup>2</sup>, 95mJ/cm<sup>2</sup>, 119mJ/cm<sup>2</sup>, 150mJ/cm<sup>2</sup>, 189mJ/cm<sup>2</sup>, 238mJ/cm<sup>2</sup> and 300mJ/cm<sup>2</sup>. Twenty-four hours later, erythema produced by these irradiations was read

using the contact Laser Doppler. This enabled comparison of the erythral response between the adapted and unadapted skin.

#### 4.2.7 Genotyping

The genotype with respect to MC1R status of all subjects was determined. This was done by automated sequencing of DNA extracted from whole blood according to previously published methods<sup>19</sup>.

### 4.3 Results

With regards to changes in thickness and melanin density obtained by Transmission Spectrophotometry, each individual's thickness and melanin density was compared with the respective value of that of the average Caucasian. This average Caucasian value was the mean thickness or mean melanosome density of the unirradiated skin of all the Caucasian subjects. In other words, the skin (irradiated and unirradiated) of each subject was compared with normal unirradiated skin of an average Caucasian person. Therefore, in precise terms, we speak of the 'relative thickness' and 'relative melanosome density'.

#### 4.3.1 Relative Thickness by UV Spectrophotometry

Following the three 'Adaptation Doses' ( $480\text{mJ/cm}^2$ ,  $520\text{mJ/cm}^2$  and  $570\text{mJ/cm}^2$ ), the thickness of the blister roofs raised in the adapted and unadapted areas was determined by Transmission Spectrophotometry. It was found that the mean thickness of unadapted and adapted skin was, respectively, 1.03 (sem 0.06) and 1.27 (sem 0.06) ( $p=0.002$ ). Examining the difference between Asians and Caucasians post-irradiation, the mean Asian skin changed in thickness from 1.17 (sem 0.12) to 1.16 (sem 0.05) ( $p=0.93$ ). In contrast, the mean Caucasian skin showed an impressive increase in thickness from 1.00 (sem 0.06) to 1.29 (sem 0.08) ( $p=0.0015$ ). This is demonstrated in Figure 1.

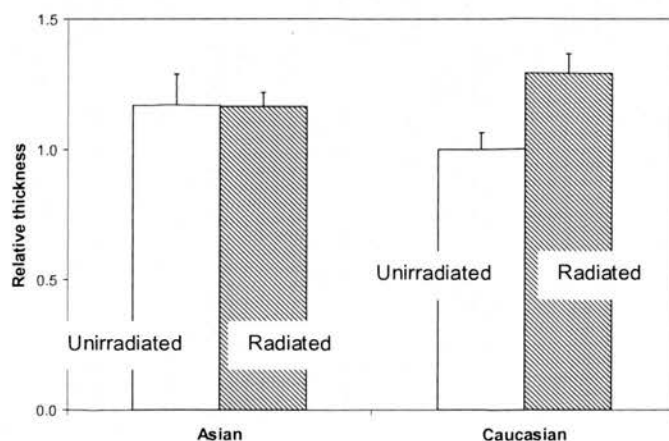


Figure 1. There was a significant increase in thickness of Caucasian skin following the three 'Adaptation Doses' of UVB.

#### 4.3.2 Relative Melanosome Density by UV Spectrophotometry

The mean melanosome density of all subjects increased following UVB irradiation. It rose from 1.4 (sem 0.25) to 1.55 (sem 0.26) ( $p=0.02$ ). Once again, the results were studied from the angle of two racially distinct groups, Asians and Caucasians. Consistent with expectation, Asian skin showed a higher baseline melanosome density than Caucasian skin. Following UVB irradiation, there was a small rise in the melanosome density in both racial groups. The melanosome density of Asian skin increased from 2.76 (sem 0.45) to 3.02 (sem 0.37) ( $p=0.10$ ), and that of Caucasian skin increased from 1.06 (sem 0.23) to 1.18 (sem 0.23) (0.09); this difference however, was not significant in either group (Figure 2).

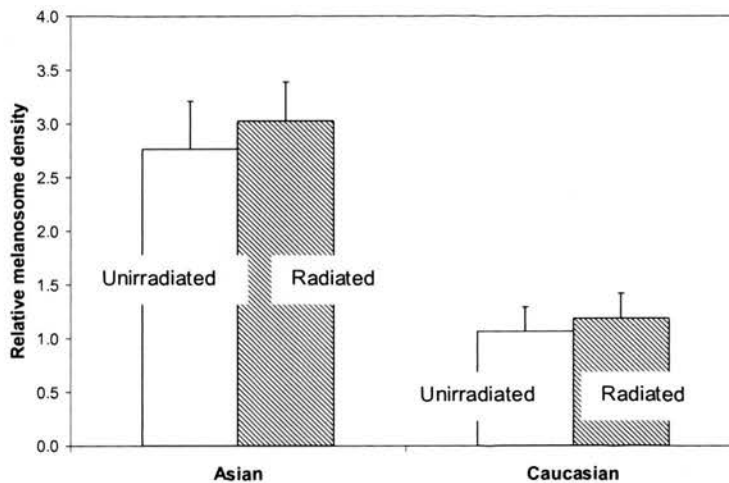


Figure 2. There was a slight increase in melanosome density of Asian and Caucasian skin following the three 'Adaptation Doses' of UVB.



The association between pigment colour (determined by  $L^*$ ) and total melanin of each unirradiated sample, relative to the reference sample, is shown in Figure 3. The x-axis represents constitutive skin colour with darker skin being represented by lower  $L^*$  scores. As expected, darker skin colour was associated with greater total melanin. Grouped regression analysis applied to datasets for Asian and Caucasian subjects showed a highly significant common slope indicated by the solid line ( $P < 0.001$ ), with no significant difference between the slopes ( $P > 0.1$ ).

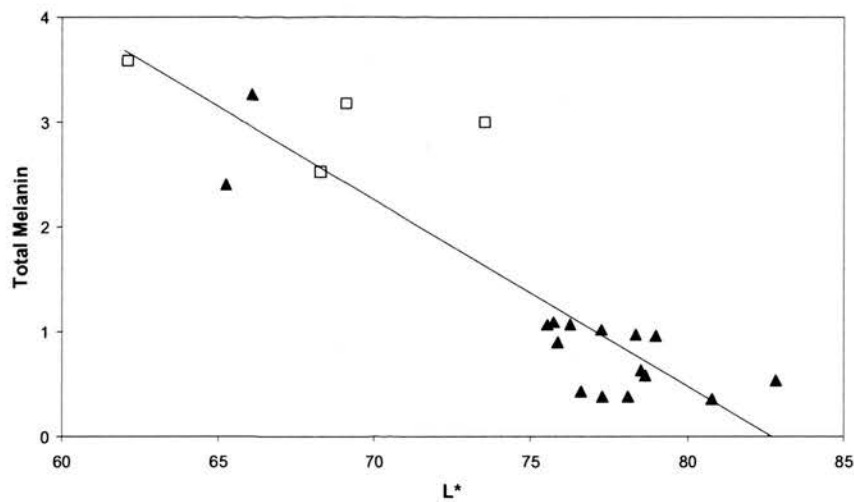


Figure 3. Darker skin colour was associated with a higher total melanin content measured by UV Transmission Spectrophotometry.  
(Squares indicate Asian subjects; triangles indicate Caucasian subjects)

#### 4.3.3 Relationship of L\* with Thickness and Melanin

The constitutive skin colour of subjects (determined by L\*) was studied against the relative thickness and total melanin before and after UVR. It can be seen that following UVR, skin thickness increased more for those with lighter skin colour, as indicated by the slope of the red line in Figure 4a. With regards to increases in total melanin however, Figure 4b shows that both, light and dark skinned subjects, showed similar increases in total melanin following UVR.

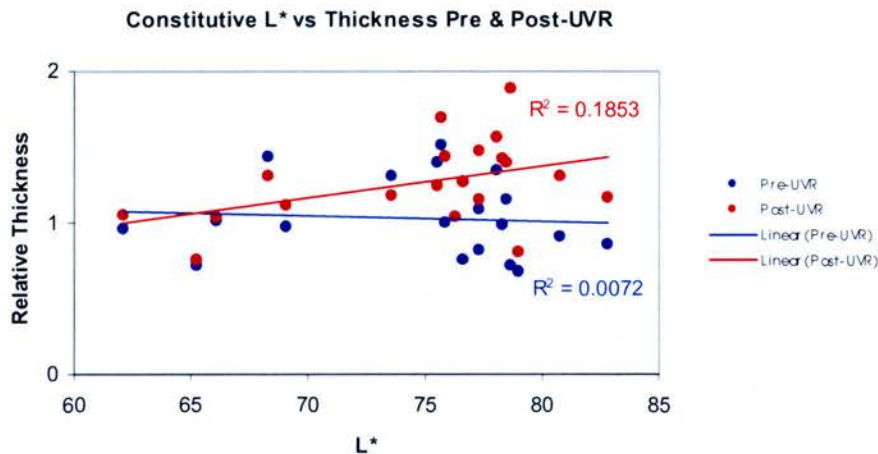


Figure 4a. Subjects with lighter skin colour showed a greater increase in skin thickness following UVR.

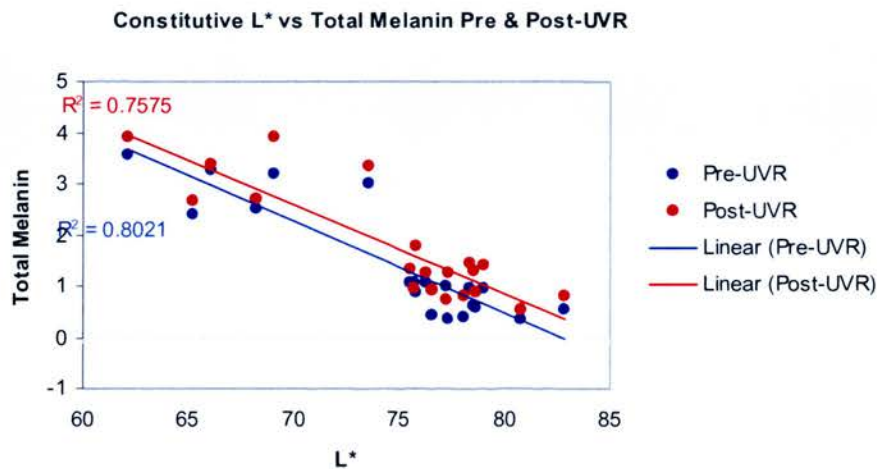


Figure 4b. Both light and dark skin colours showed an increase in total melanin following UVR.

The overall picture demonstrates that following UVR, there was an increase in skin thickness and melanosome density, as determined by UV Spectrophotometry, together with an increase in darkness of skin colour as shown in Figure 5.

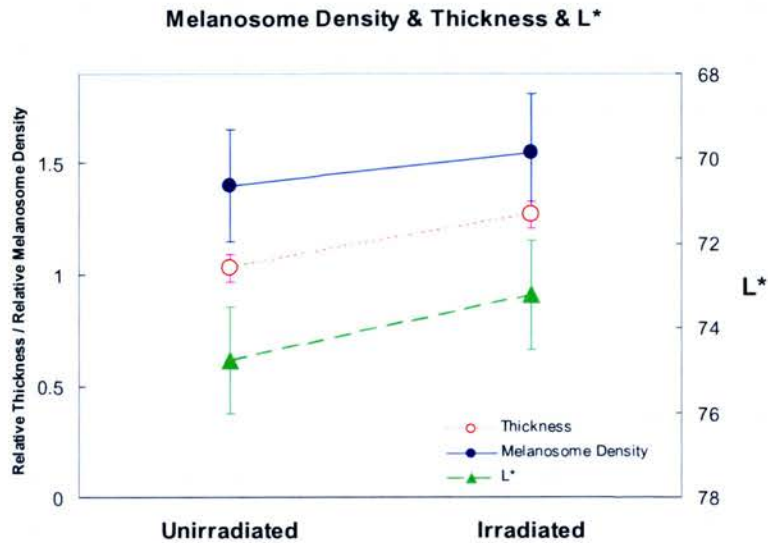


Figure 5. Following UVR, there was an increase in melanosome density, thickness and skin darkness ( $L^*$ ).

#### 4.3.4 Measuring Photoprotection

As mentioned earlier in Section 2.1 (Methods), 16 of the 20 volunteers were exposed to a series of UVB on the left (unadapted) and right (adapted) lower backs at the time of blister harvesting. The purpose of this step was to assess the photoadaptive response of the irradiated area of skin at the exact timepoint during which the blister roofs were obtained, and relate this response to changes in skin thickness and melanin at that time.

It was found that there was a significant reduction in the degree of Doppler flux of the adapted skin following set doses of UVB (Figure 6). For the highest dose of UVB delivered ( $300\text{mJ}/\text{cm}^2$ ), the flux of the adapted site and unadapted site was, respectively, 236.64 (sem 32.29) and 99.43 (sem 18.71) ( $p < 0.05$ ). Overall, across all doses, there was a mean reduction in flux of 66.5% (sem 7.61) on the adapted site compared with the unadapted site.

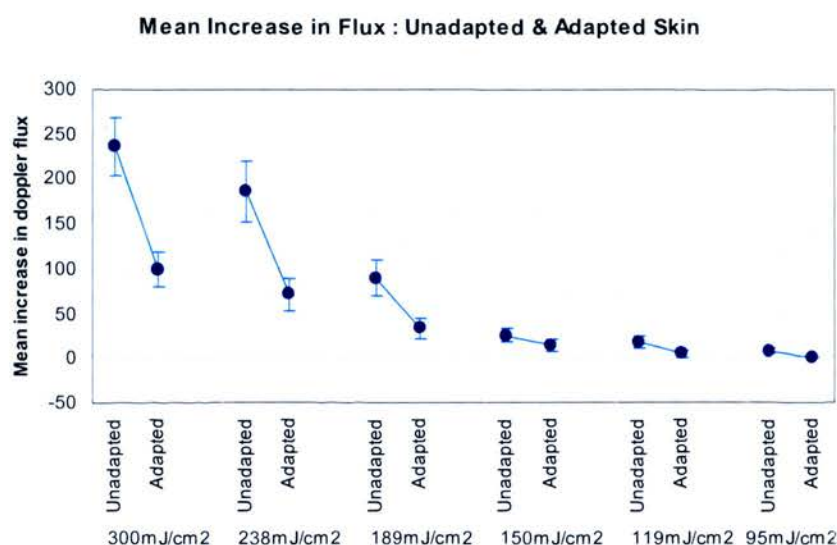


Figure 6. There was a decrease in flux on the adapted skin at the time of blister harvesting, showing that there was a level of photoadaptation on the previously irradiated site.

#### 4.3.5 Quantification of Eumelanin and Pheomelanin Concentrations by Chemical Assay

As mentioned in Chapter 2, once the blister roofs were subjected to UV Transmission Spectrophotometry to determine its absorbance characteristics, the blister roofs were subjected to HPLC assays for quantification of eumelanin and pheomelanin. 19 samples were processed as such, and the results showed a mean increase in both eumelanin and pheomelanin concentrations. Figure 7 shows the increase in eumelanin and pheomelanin concentrations following UVB.

The dataset comprises Europeans and Asians. Following irradiation, the eumelanin concentration in Asian skin increased from 4668ng/mg (sem 609) to 7212ng/mg (sem 1094) ( $p=0.03$ ). For Caucasians, the eumelanin concentration increased from 1774ng/mg (sem 465) to 2229ng/mg (sem 476) ( $p=0.03$ ). Pheomelanin levels in Asian skin rose from 290ng/mg (sem 77) to 462ng/mg (sem 129) ( $p=0.05$ ), and in Caucasian skin, pheomelanin levels increased from 123ng/mg (sem 26) to 145ng/mg (sem 21) ( $p=0.1$ ). As would be expected, there was a significant difference in the baseline eumelanin levels between Asians and Caucasians ( $p=0.007$ ); baseline pheomelanin levels, however, were not significantly different between the races ( $p=0.11$ ). Following irradiation, all subjects, except three, showed an increase in both eumelanin and pheomelanin.

With regard to the eumelanin:pheomelanin ratio, there was no significant racial difference in the eumelanin:pheomelanin ratios. Caucasian and Asian eumelanin:pheomelanin ratios are respectively: 15.48 (sem 1.96) and 18.82 (sem 3.82) ( $p=0.5$ ).

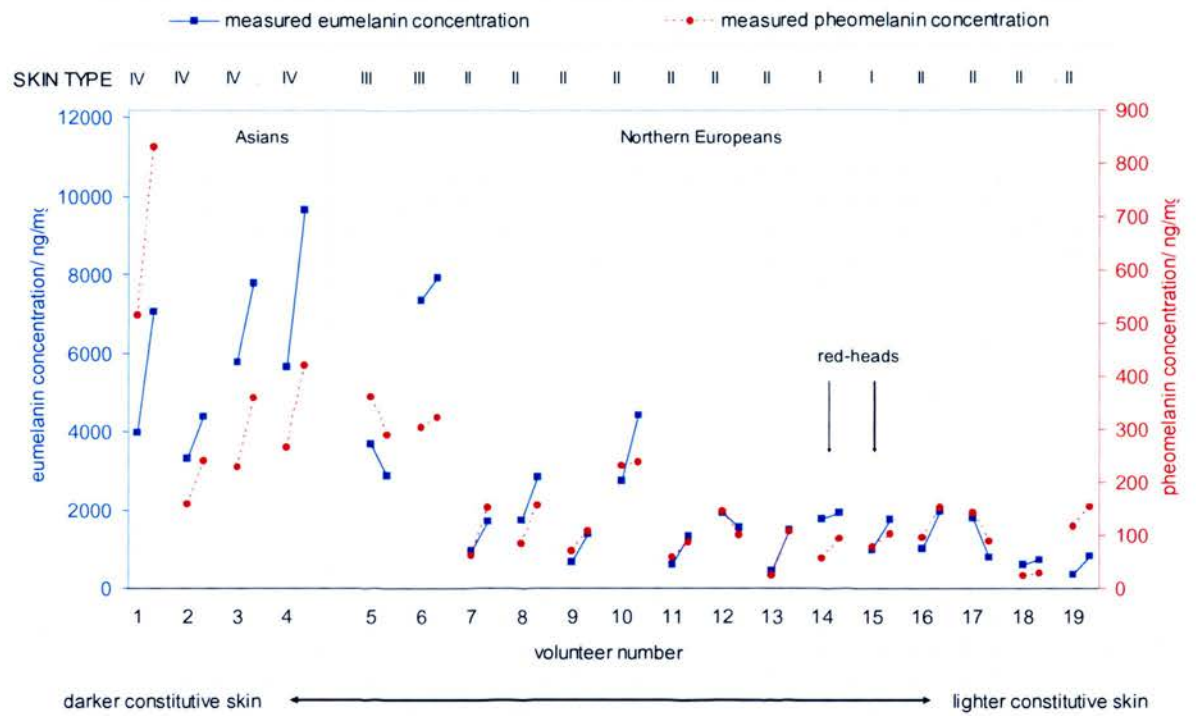


Figure 7. Eumelanin and pheomelanin levels before and after UVB irradiation for Asian and Caucasian subjects. Results are plotted in linked pairs for each individual, with unirradiated skin on the left of each linked pair.



#### 4.3.6 Relationship between L\* and Melanin Concentration

The relationship between L\* and melanin concentration was examined and is detailed in Figure 8. In both constitutive and irradiated skin, there was good correlation between L\* and each of the melanin types. The darker the L\*, the greater was the melanin concentration. There also existed a positive correlation between eumelanin and pheomelanin in both constitutive and irradiated skin.

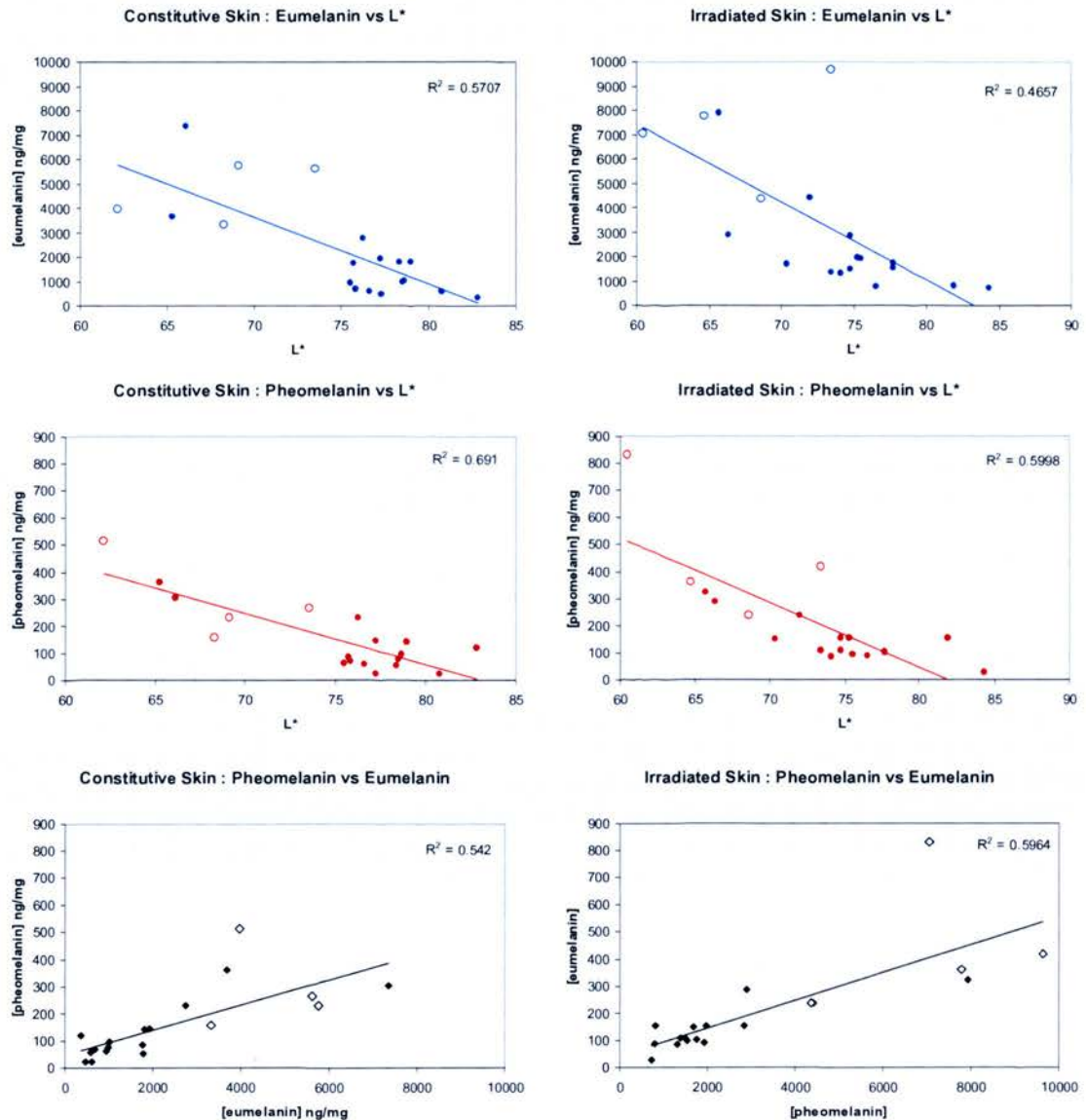
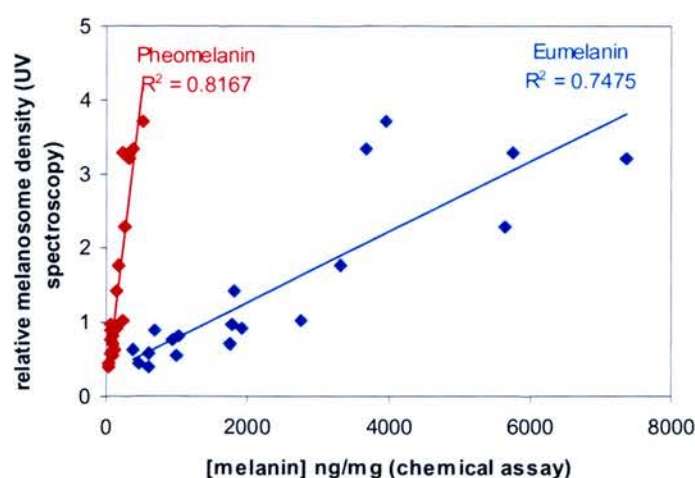


Figure 8. In both constitutive and irradiated skin, there is a positive correlation between darker skin colour and melanin concentration (eumelanin and pheomelanin). There is also a positive correlation between eumelanin and pheomelanin concentrations. The open symbols represent Asian subjects; closed symbols represent Caucasian subjects.

#### 4.3.7 UV Spectrophotometry vs Chemical Assay

Having determined the concentration of melanin using two distinctly different methods, it is of interest to note if both methods are in agreement with regard to their individual findings. Figure 9 shows the correlation between UV Transmission Spectrophotometry and the HPLC Chemical Assay in determining melanin concentrations. In both constitutive and irradiated skin, it is reassuring to find that there is good correlation between the relative melanin density, as determined by UV Spectrophotometry, and the concentration of melanins, as determined by HPLC.

##### a) Constitutive Melanin : Spectroscopy vs Chemical Assay



##### b) Irradiated Skin : Spectroscopy vs Chemical Assay

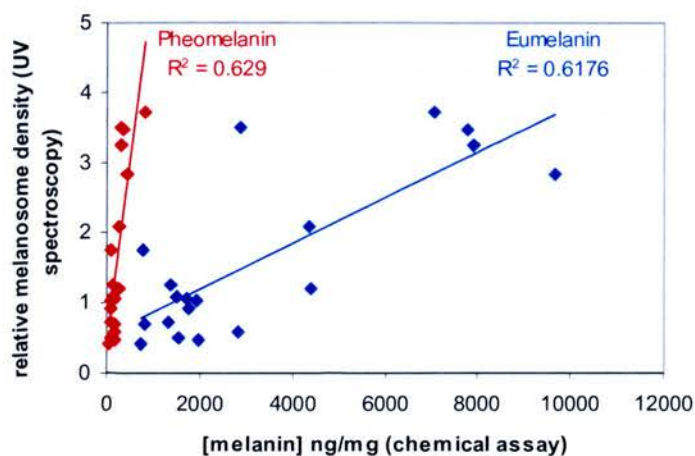


Figure 9a) & 9b). In both constitutive and irradiated skin, there is a positive correlation between the two methods of melanin determination: UV Transmission Spectrophotometry and HPLC Chemical Assay.

#### 4.4 Discussion

The main purpose of UV Transmission Spectrophotometry was to quantify epidermal thickness and melanin density. These variables are part of the human photoadaptive response to UVR and therefore, they change following irradiation. In this study, following UVB, there was a significant increase in epidermal thickness. Looking at the group as a whole, the epidermis was thicker by 1.27 times following photoadaptation with UVB. This is compared to unadapted epidermis whose figure is 1.03 (both these figures are relative to a baseline group of Caucasian subjects whose thicknesses are taken as the arbitrary baseline). When the group was split according to racial background, thickening of the epidermis was more pronounced in Caucasians; there was a significant difference between control unadapted skin and irradiated skin in the Caucasian group of subjects. In Asian skin, however, there was no significant increase in thickness. This may perhaps be due to the fact that Asian skin has a higher baseline melanin level, therefore, the photoadaptive response from thickening may not be activated at the lower doses which stimulate hyperplasia in Caucasian skin. For future studies, it is worthwhile investigating this response of epidermal thickening in Asians with higher doses of UVR. Nevertheless, when studied as a group, there is no doubt that following our regime of photoadapting the skin with UVB radiation, there was significant thickening of the epidermis. This confirms our suspicion that besides an increase in melanin, there is another mechanism serving to protect the skin from subsequent and future UV injury.

In studying the photoadaptive response afforded by melanin, it was found that as a whole, there was a significant increase in the melanosome density following irradiation. When the dataset was split into two racial groups (Asians and Caucasians), the rise in melanosome density was small and the difference was not significant. Keeping in mind that melanosome density takes into account the thickness of the epidermis, and as shown here, thickness increases following UVR, melanosome density as a measure may underestimate total melanosome production. Future studies should call for a larger group of volunteers with a greater number of Asian subjects than this study. A larger UVB dose should be trialed, as this may perhaps evoke a greater pigmentary response, particularly in the Asian subjects.

The reason why a standard irradiating dose was chosen for all subjects instead of using MEDs (minimal erythral doses), was to see how subjects of different skin phototypes reacted to the same standard dose. We expected that using the one standard dose across the board would separate apart our participants' responses as widely as possible, according to their skin phototype. We did not seek to observe the reaction to a biologically equivalent dose, but instead, sought to observe how different people of varying skin colours reacted to the one absolute dose. In reality, if a group of people were to step outdoors into sunlight, they would all be exposed to the same dose, which would not be biologically equivalent for all of them. We hoped for the study to be closer to reality. The disadvantage of using a standard dose instead of MEDs is that since the dose is not biologically equivalent, the

response from subjects of darker skin is less pronounced compared to lighter skinned subjects. Consequently, one may not achieve the results which one would have hoped for at the outset. In this study, this is highlighted in our Asian subjects experiencing less skin thickening; it is suspected that this is because the dose which they received is not biologically equivalent and as potent to that received by the Caucasian subjects.

The pigment colour measured by  $L^*$  using the Minolta Chromameter showed a positive correlation with melanosome density obtained by UV Transmission Spectrophotometry, as would be expected. This confirms the use of  $L^*$  as a valid and reliable non-invasive measure for pigment colour due to melanin.

Getting to the heart of the question, does the increase in epidermal thickness and melanin density translate to photoprotection? The results showed that following several adapting doses of UVB to the back, a future UVB challenge to the area resulted in less erythema than is produced on unadapted control skin. The reduction in erythema (measured as flux) was significant. We know that this level of photoprotection is within the individual blister roof samples, as they were harvested on the very day of the challenge. Therefore, the photoprotection in the adapted skin can be related to the increase in epidermal thickness and melanosome density.

In addition to UV Transmission Spectrophotometry, the quantification of melanin was also determined by chemical degradation, which yielded information on specific quantities of eumelanin and pheomelanin in the blister samples. As expected, Asian skin contained higher baseline eumelanin levels, however their pheomelanin levels were not shown to be different to that of Caucasian skin. It is suspected that this may be due to the small sample size of Asian subjects ( $n=4$ ) in our study. Following UVB, both eumelanin and pheomelanin increased significantly in both racial groups but there was no significant difference in the eumelanin:pheomelanin ratio before and after UVR. A further question is whether there is a difference in the eumelanin:pheomelanin ratio between racial groups. It was found that the eumelanin:pheomelanin ratio was the same in Asians and Caucasians; once again, a larger sample size of Asian subjects would be more confirmatory of this result.

Once again with regard to the chemical assay, as with UV Transmission Spectrophotometry, there was good correlation between  $L^*$  and both melanin types, (keeping in mind that eumelanin levels are over tenfold greater than pheomelanin), confirming  $L^*$  as a good clinical measure of pigment colour.

Despite the two methods being distinctly different, UV Transmission Spectrophotometry and HPLC produced similar results with regard to constitutive and facultative melanin levels, as well as between racial types. There is good correlation in melanin concentrations obtained by UV Transmission Spectrophotometry and HPLC, confirming the validity of Spectrophotometry in the quantification of

melanin. An added advantage of UV Transmission Spectrophotometry is its ability to provide information not only on melanin levels, but also epidermal thickness.

This study was highly intensive. Its main purpose was to separate or 'tease out' the components of the human photoadaptive response: epidermal thickening and melanin production. The earlier Chapter 2 examined photoadaptation as a whole, encompassing both these components. Chapter 3 then examined how pigmentation increased following UVR. It was the aim of this current chapter to investigate if the human epidermis thickens at all following UVR and if it does, to quantify this response. With UV Transmission Spectrophotometry, it was found that there is a convincing increase in thickness of human epidermis following UVR. The increase in epidermis is associated with greater photoprotection, as demonstrated by less UV-induced erythema being suffered by the thicker skin. This confirms earlier suspicions that there is an alternative photoprotective mechanism in human skin apart from melanin production. The increase in epidermis is also shown to vary according to racial background, with lighter-skinned Caucasian subjects experiencing greater increases in epidermal thickness compared to darker-skinned Asian subjects. An improvement in this study would be to have a wider range of skin colours, and greater numbers of Asian subjects. It would be interesting to investigate if epidermal thickness can occur to the same degree in subjects of varying skin colours, if the appropriate biologically potent UV dose was to be given – how does this dose relate to the MED? Is epidermal thickening more predominant in lighter-skinned subjects, assuming that darker-skinned subjects photoprotect initially with melanin production. These are questions that still need answering in future studies. In addition to quantifying epidermal thickness, UV Transmission Spectrophotometry was also able to quantify indirectly, the increase in skin melanin. As expected, this was also shown to increase following UVR. These increases in thickness and melanin were able to be related to visual and clinical observations of the photoadaptive response. Chemical degradation assays further confirmed findings on the indirect calculations of the melanin levels before and after UVR.

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## Chapter 5 \* Vitiligo Study

### 5.1 Introduction

This study aims to explore the photoadaptive response of the skin in vitiligo. In the condition of vitiligo, melanocytes may be decreased in number or altogether absent<sup>1,2,3,4</sup>. Therefore, lesions of vitiligo lack the photoprotection lent by melanin. The question we would like to ask is whether the skin in vitiligo demonstrates an alternative way of photoprotection, ie. through epidermal thickening. We speculate that this may be so, and investigate if this is true. In conducting this experiment, we looked at several points:

- 1) the difference in melanin concentration between vitiligo skin and normal skin.
- 2) the difference in epidermal thickness between vitiligo skin and normal skin.
- 3) the difference in photoprotection between vitiligo skin and normal skin in response to UVB radiation.

Histopathologically, the skin in vitiligo is different to normal skin in that there is an absence of melanocytes, increased numbers of inflammatory cells and epidermal vacuolization with thickening of the basement membrane<sup>1</sup>; inflammatory changes are seen even in stable longstanding vitiligo<sup>2</sup>. It is debatable whether melanocytes are absent or only reduced in number in vitiligo lesions; immunohistochemical studies have found melanocytes to be lost or absent in vitiligo lesions<sup>3</sup>. However, there is evidence of melanocyte survival on molecular analysis of longstanding vitiligo lesions<sup>4</sup>.

It has been shown that there is less photoprotection in vitiligo; experiments have found that a lower dose of UVB is needed to produce the same level of erythema in vitiligo as in normal pigmented skin<sup>5</sup>. Vitiligo skin suffers greater UV injury following exposure to UVB, however its rate of repair is similar to normal skin<sup>6</sup>. Experiments have shown that following UV exposure to vitiligo skin, erythema and hyperkeratosis occur to a greater extent than on normal pigmented skin; this increase in thickness of the epidermis can be seen histologically<sup>7</sup>. In vitiligo, the stratum corneum is thought to play a major role in photoprotection, as it is thicker than on the adjacent normal pigmented skin within the same person<sup>8</sup>.

Therefore the main reason for this study was to investigate for any innate photoprotection in vitiligo skin, given its lack of melanin. The difference in thickness between vitiligo and normal skin was closely examined. We wonder if any increase in thickness in vitiligo skin confers a level of photoprotection and how this level of photoprotection in vitiligo skin compares with that in normal pigmented skin.

## 5.2 Methods

Ten subjects with vitiligo were recruited from the general Edinburgh population; the colour of their vitiligo and normal skin was measured. Nine of the ten subjects had the melanin concentrations of their vitiligo and normal skin measured by a chemical degradation assay. The epidermal thickness of 8 of the subjects was measured using UV Transmission Spectrophotometry (detailed in Chapter 1 and Chapter 4). Out of the 10 subjects, 7 subjects were given 3 doses of UVB to an area of vitiligo and an adjacent area of normal pigmented skin. This was done in order to measure and compare the photoprotective level of vitiligo and normal skin.

### 5.2.1 Skin Colour Measurements

The skin colour of vitiligo and normal skin of all 10 subjects was measured with the Minolta Chromameter using the  $L^*a^*b^*$  system. For each colour measurement of a vitiligo patch, a directly adjacent patch of normal skin was also measured for comparison; any anatomical variation was minimised by having the two patches directly adjacent to each other. The body sites of vitiligo for each subject is shown in Table 1. Colour measurements on the  $L^*$  axis were taken before and after noradrenaline iontophoresis of both, normal and vitiligo skin. This was performed in order to eliminate the influence of blood or erythema from the pigmentation colour measurements.

Vitiligo Site	Skin Type	Racial Type
abdomen	II	Caucasian
forearm	II	Caucasian
forearm	III	Caucasian
forearm	III	Caucasian
abdomen	III	Caucasian
abdomen	III	Caucasian
forearm	III	Caucasian
chest	III	Caucasian
upper back	V	Asian-Caucasian
lower leg	V	Middle Eastern

Table 1. Body sites that were studied on subjects with vitiligo.

### **5.2.2 Melanin Concentration**

The eumelanin and pheomelanin concentrations of normal and vitiligo skin were measured by a chemical degradation assay. The skin samples were obtained from 9 subjects by suction blistering (refer to Chapter 1). For each subject, a blister was taken from a patch of vitiligo, and another blister from the adjacent normal skin. The samples were freeze-dried prior to being subjected to a chemical degradation assay<sup>9</sup>. Chemical degradation of eumelanin by acidic permanganate oxidation formed pyrrole-2,3,5-tricarboxylic acid (PTCA); hydriodic acid reductive hydrolysis of pheomelanin formed 4-amino-3-hydroxyphenylalanine (4-AHP). The concentrations of these degradation products were then determined using HPLC assays; eumelanin and pheomelanin concentrations were then obtained by multiplying by factors of 160 for eumelanin, and 9 for pheomelanin.

### **5.2.3 Skin Thickness Measurements**

Prior to the quantification of melanin, the epidermal thickness of 8 of the skin samples was measured by UV Transmission Spectrophotometry using the Fluoromax-3 Spectrophotometer, operated in synchronous scanning mode. The skin samples were placed directly in front of the entrance aperture of an integrating sphere which collects all forward transmitted and scattered radiation. The spectral transmittance (relative to a cell containing only saline) was obtained over the spectral region 250-400nm in 1nm steps; the spectral absorbance was derived as the logarithm (to base 10) of the reciprocal of spectral transmittance.

This method, also detailed in the previous chapter, allowed for comparison of the absorbances of normal and vitiligo skin. The absorbance of normal skin is due to i) melanin, ii) biomolecules (which relates to thickness) and iii) scattering. In vitiligo skin, absorbance by any melanin at all is negligible, therefore absorbance is due only to biomolecules and scattering. Once each component is accounted for, a comparison can be made between normal and vitiligo skin with regards to the absorbances due to biomolecules, therefore information on the thickness of each is gained.

### **5.2.4 Measuring Photoadaptation: UVB Challenge**

In order to compare the difference in photoprotection of vitiligo and normal skin, 7 subjects were given three set doses of UVB to an area of vitiligo; the same three doses were also delivered to an adjacent area of normal skin. The doses, delivered using the Xenon Arc Lamp, in spots of 1 cm in diameter, were 28mJ/cm<sup>2</sup>, 56mJ/cm<sup>2</sup> and 112mJ/cm<sup>2</sup>. Figure 1 is a pictorial representation of the doses delivered. Twenty four hours following delivery of the UVB doses, erythema was measured using the contact laser Doppler. Three measurements were taken and a mean of the three taken.

The main reason why the Xenon Arc Lamp was used here, and not the Diffey Lamp or the Waldmann TL-01, is that the Xenon Arc Lamp allows flexibility on the placement of irradiation spots on the skin. As vitiligo patches are irregular in shape and size, it was important that the irradiating instrument be

flexible enough to allow various arrangements of irradiating doses along the border between vitiligo and normal skin.

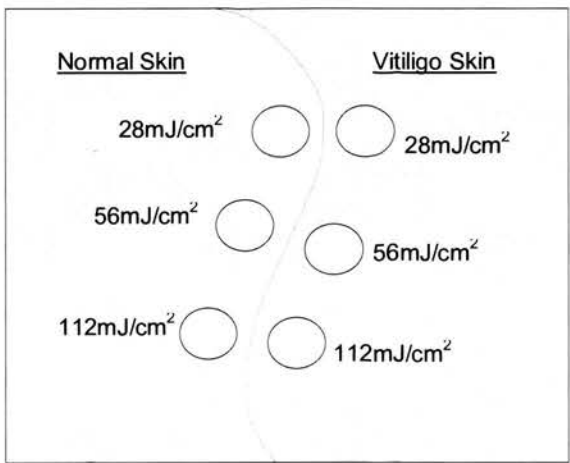


Figure 1. A pictorial representation of how the doses were delivered on vitiligo skin and the adjacent normal skin.

5.3 Results

5.3.1 Skin Colour Measurements

The skin colour of all 10 subjects was measured using L\*. As expected, the vitiligo skin of all subjects registered a higher L\* value (lighter colour) than normal skin. Figure 2 shows the colour of normal and vitiligo skin for all subjects. The mean L\* of normal skin was 69.51 (sem 2.37), and that of vitiligo skin, 78.40 (sem 1.01) (p=0.001).

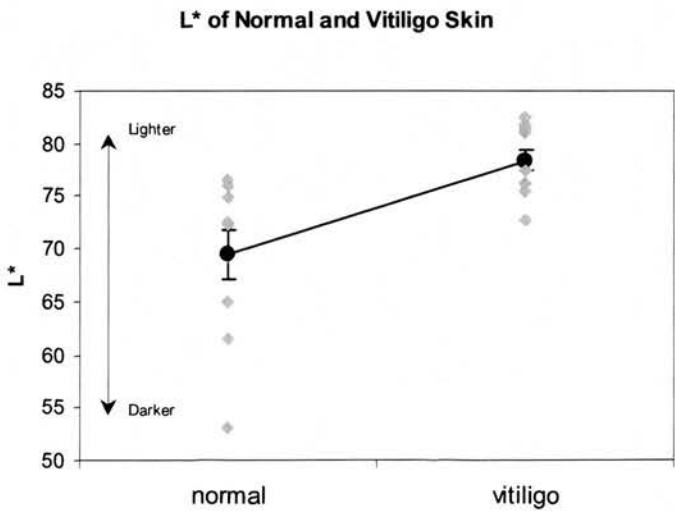


Figure 2. Vitiligo skin registers a higher L\* value (lighter) than normal skin.



5.3.2 Melanin Concentration by Chemical Assay

The eumelanin and pheomelanin concentrations of both normal and vitiligo skin was measured for 9 of the volunteers. Of the vitiligo samples, the eumelanin concentration of 6 of the 9 volunteers was undetectable by the assay ( $< 96\text{ng/mg}$ ) and pheomelanin concentration of 5 of the 9 volunteers was undetectable by the assay ( $< 19\text{ng/mg}$ ). The highest eumelanin concentration found in vitiligo skin was  $410\text{ng/mg}$  and this came from a mixed Asian-Caucasian volunteer whose normal skin had a eumelanin concentration of  $13,584\text{ng/mg}$ ; this volunteer had the highest normal skin eumelanin and pheomelanin concentration of the whole group. The highest pheomelanin concentration in vitiligo skin was  $50\text{ng/mg}$  from a Caucasian volunteer, whose normal pheomelanin concentration was  $133\text{ng/mg}$ . Figure 3 shows the eumelanin and pheomelanin of each volunteer, plotted in linked pairs. Each linked pair shows the normal skin on the left and vitiligo skin on the right of the link. Figure 4 shows the mean melanin concentrations of vitiligo skin and normal skin. The mean eumelanin concentration in normal and vitiligo skin is, respectively,  $3957\text{ng/mg}$  (sem 1295) and  $150\text{ng/mg}$  (sem 51.39) ( $p=0.02$ ). The mean pheomelanin concentration in normal and vitiligo skin is, respectively,  $267\text{ng/mg}$  (sem 56.96) and  $14$  (sem 6.94) ( $p=0.002$ ).

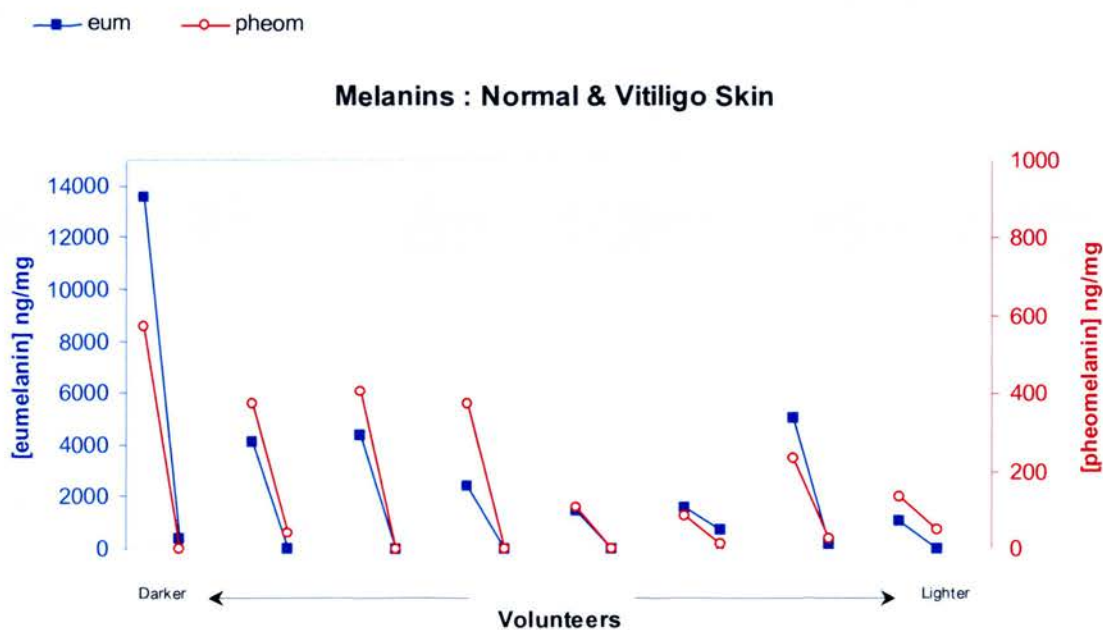


Figure 3. Eumelanin and pheomelanin concentrations of the subjects shown in linked pairs. Normal skin is to the left of each link, and vitiligo skin to the right.

### Mean Melanins : Normal & Vitiligo

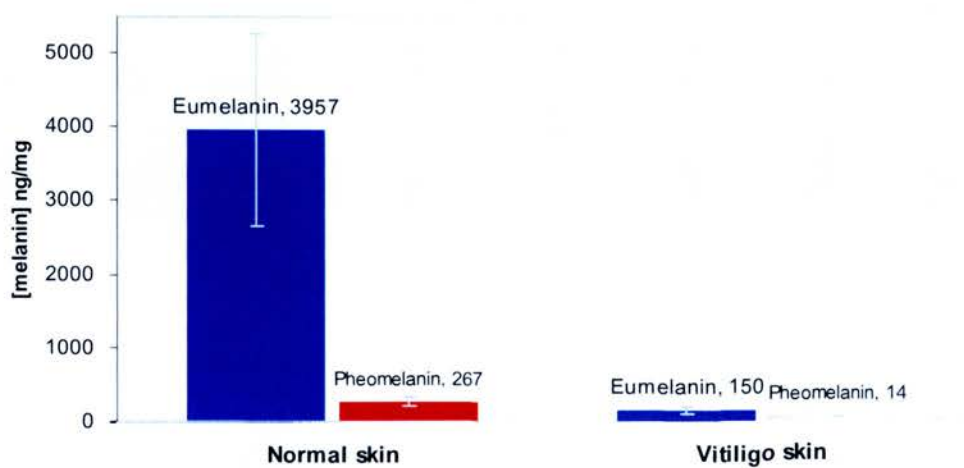


Figure 4. There is a significant difference in the mean melanin concentrations between normal skin and vitiligo skin.

#### 5.3.3 Thickness Measurements

The epidermal thickness of the 8 normal skin samples and 8 vitiligo skin samples was determined using UV Transmission Spectrophotometry. The results are presented in the form of the relative thickness of the vitiligo skin to the adjacent normal skin of the given volunteer. For all samples, the vitiligo skin was either the same as, or thicker than the adjacent normal skin. Figure 5 shows the relative thickness of each subject's vitiligo skin, in the order of skin darkness ( $L^*$ ). The mean relative thickness of vitiligo skin is 1.23 (sem 0.12). Figure 5 shows a correlation between constitutive skin colour and the relative thickness of vitiligo epidermis, it appears that the darker the constitutive skin colour of a subject, the thicker is his vitiligo skin relative to his normal skin ( $R^2 = 0.8104$ ). However it is important to note that the slope of the graph in Figure 5 is highly dependent on the individual located on the far right of the graph. This individual had the greatest relative thickness of 2; this individual was also of the darkest constitutive skin colour ( $L^* 53.13$ ) For future studies, a greater number of individuals of darker skin colour are needed to show if there is a strong correlation between thicker vitiligo skin and constitutive skin colour. There was no correlation between relative thickness and  $L^*$  of the vitiligo skin itself.

### Relative Epidermal Thickness of Vitiligo Skin

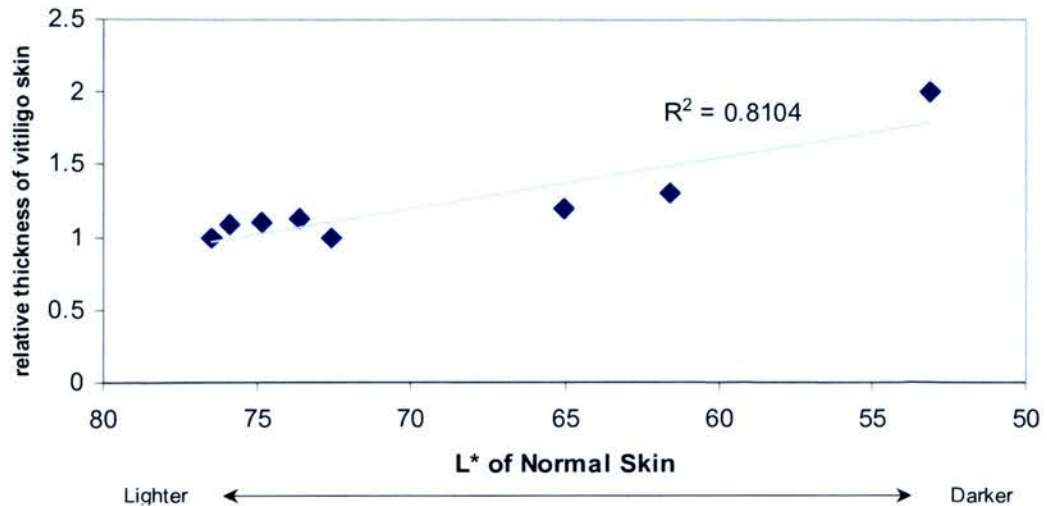


Figure 5. The relative thickness of vitiligo skin was greatest for subjects whose constitutive skin colour was darker. However, the individual located on the far right has a great bearing on the slope of the graph.

#### 5.3.4 Measuring Photoadaptation: UVB Challenge

Both the vitiligo and adjacent normal skin of 7 of the volunteers were subjected to a challenge with UVB doses of 28mJ/cm<sup>2</sup>, 56mJ/cm<sup>2</sup> and 112mJ/cm<sup>2</sup>; erythema measured as an increase in flux produced by each dose was measured with the Laser Doppler, 24 hours following the challenge. For each dose, the mean increase in flux of the vitiligo skin was higher than that of the normal skin. This was most pronounced for the highest dose. Following 112mJ/cm<sup>2</sup>, the mean increase in flux on areas of normal and vitiligo were respectively, 99.76 (sem 34.73) and 305.86 (sem 91.62) (p=0.02). Following 56mJ/cm<sup>2</sup>, the values for normal and vitiligo skin was respectively, 11.56 (sem 6.67) and 47.72 (sem 13.36) (p=0.04). Finally, for the lowest dose of 28mJ/cm<sup>2</sup>, the values for normal and vitiligo skin were respectively, 1.11 (sem 2.32) and 8.11 (sem 3.97) (p=0.3). This is shown graphically in Figure 6.



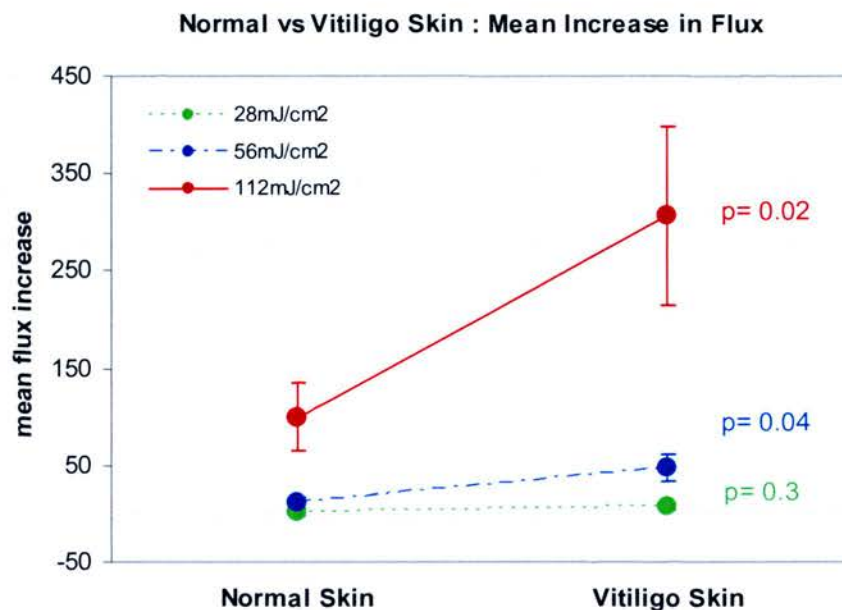


Figure 6. Following a UVB challenge of three different doses, vitiligo skin showed a greater flux increase than normal skin, this translates as less photoprotection in vitiligo skin.

#### 5.4 Discussion

The aim of this study was to examine the melanin content and epidermal thickness of vitiligo skin, and relate these variables to the photoprotection or its lack thereof in vitiligo. It was shown by non-invasive measurements of pigmentation colour that, as expected, vitiligo skin is paler or lighter than adjacent healthy skin. Quantification of eumelanin and pheomelanin by a chemical assay further confirmed the difference in melanin concentrations between vitiligo and normal healthy skin. The Caucasian vitiligo skin was shown to have either immeasurable quantities of melanin (equating to no melanin by chemical assay) or very little melanin. ‘Very little melanin’ falls in the mean region of 117ng/mg for eumelanin, a value significantly different to normal Caucasian skin, whose melanin concentration is over 23-fold this value.

Vitiligo skin was found to be either thicker than, or the same as surrounding normal skin. There is also a positive correlation between the thickness of the subject’s vitiligo skin and his/her constitutive pigmentation. This makes good sense, as one would expect for darker skin to have greater photoprotection by melanin; thus a patch of vitiligo in a darker person would be forced to compensate by thickening to a greater degree (in order to ‘keep up’ with the surrounding high level of photoprotection) than in a lighter person. Looking at the matter from a different angle, one could say

that the surrounding normal dark skin does not need to thicken as much as the vitiligo skin to gain the same level of photoprotection. In other words, the less different is the pigmentation between normal and vitiligo skin, the more similar will be their thicknesses. This suggests that epidermal thickening is one of the mechanisms by which individuals of lighter skin colour protect themselves from UV injury; this seems to be more important in those with less melanin.

So, how effective is the mechanism of epidermal hyperplasia in protecting one from UVR damage? In the attempt to answer this question, the photoprotective capacity of vitiligo skin was examined clinically by UVB challenge doses. As expected, the photoprotective level of vitiligo skin was less than that of healthy normal skin. Despite the fact that vitiligo skin is, on average, thicker than surrounding normal skin, UV insult to vitiligo skin imparts a greater erythematous response. This suggests that despite epidermal hyperplasia in vitiligo skin, the lack of melanin accounts, to a large degree, for the lack of photoprotection in vitiligo.

This was a neat study which examined photoprotection due to epidermal hyperplasia in skin devoid of melanin. Improvements can be made in the future based on this current study. Our sample size was small; a larger sample size would enable the study to be split into subgroups based on body areas. The clinical skin colour of majority of our subjects measured by L\* is clustered around 70-80 (Caucasian light skin); a wider racial spectrum with L\* down to 30-40 (African/South Asian) would be valuable in future studies. A further point worthy of study is the difference in vitiligo skin from photoprotected and photoexposed areas. It is predicted that photoprotected vitiligo skin may not demonstrate the same degree of epidermal hyperplasia if at all, when compared with vitiligo skin in photoexposed areas.

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## Conclusion

The last four chapters have described, in detail, experiments investigating the photoadaptive response of human skin to ultraviolet radiation. It is understood that, following UVR, the human skin developed a tolerance which served to protect it from further UV damage. This protection is gained by two major factors: the development of pigmentation / melanin, and the thickening of the epidermis. From these studies, it is understood that pigmentation is an extremely important mechanism in the photoadaptive response. It was shown to peak at 1 week following UV exposure, and gradually waned over the next 12 weeks. How long pigmentation lasts in an individual depends on their constitutive pigmentation. Our study showed that fair-skinned, redheaded subjects pigmented to a lesser degree when compared with darker-skinned Asian subjects. Interestingly, the rate of pigmentation loss, or the time to return to one's constitutive pigmentation level, is the same in both the fair-skinned redhead and the darker-skinned Asian. The intensity or degree to which an individual pigments also depends on the dose of UV exposure. These experiments demonstrated a positive dose-response, with higher doses of UVR producing a darker pigmentation colour.

In measuring pigmentation colour, the new method employed, using iontophoresis of noradrenaline, allows pigmentation colour to be assessed without the influence of erythema. This method successfully suppressed UV-induced erythema at all timepoints except for the first 24 hours post UV exposure. Prior to this study, iontophoresis of noradrenaline for measurement of pigmentation colour had not been performed by any other investigator. It is hoped that this method will be more widely used; although slightly laborious, it is non-invasive and reproducible. This clinical method of measuring pigmentation with  $L^*$  is supported by chemical assay methods measuring the concentrations of eumelanin and pheomelanin. As expected, following UVR, both eumelanin and pheomelanin concentrations increased. Asian subjects showed a greater increase in both melanins compared with Caucasians.

Epidermal hyperplasia is another mechanism by which the skin adapts to UVR. From these experiments, it was found that the epidermis increased in thickness following several doses of UVR. This was, of course, accompanied by hyperpigmentation. UV Transmission Spectrophotometry not only allowed for measurements of epidermal thickness, but also melanin density. Melanin density obtained by this method correlated positively with melanin concentrations obtained by a known chemical assay. Reassuringly, both these methods also correlated with  $L^*$ , which has been used throughout as the clinical measure of pigmentation darkness. Darker  $L^*$  values correlated with a higher melanin density (by UV Transmission Spectrophotometry), as well as higher eumelanin and pheomelanin concentrations (by chemical assay). With regards to epidermal hyperplasia, the difference in racial types is highlighted by the greater degree of epidermal hyperplasia in lighter-skinned Caucasians when compared with Asians. Although the sample size was small, the results make

intuitive sense in that, the lighter the skin colour of an individual, the greater is the response of the epidermis in thickening following UV exposure.

In the condition of vitiligo where there is a lack of melanin and pigmentary capacity, it is shown that vitiligo patches are thicker than normal surrounding pigmented skin. In this study, it was firstly confirmed by epidermal samples subjected to a known chemical assay, that eumelanin and pheomelanin levels were significantly reduced in vitiligo skin. With regards to epidermal hyperplasia of vitiligo patches, it is interesting to note that those of darker constitutive skin colour had vitiligo patches with greater epidermal thickness (the comparison is made between vitiligo skin and normal pigmented skin within the same subject). Despite the epidermal hyperplasia, patches of vitiligo still burnt more than normal surrounding pigmented skin when both were given the same dose of UVR, meaning that despite the increased thickness, vitiligo skin is still lacking in photoprotection, compared to normal pigmented skin. This translates to mean that the pigmentary component of the photoadaptive response is vital in protecting the skin from UV insult; epidermal hyperplasia on its own does not suffice in providing adequate photoprotection.

From these studies it is understood that the photoadaptive response as a whole lasts several weeks. The study examining the photoadaptive response as a whole was based on a regime of three photoadapting doses over three consecutive days. It is suspected that experiments employing different regimes will yield different results on the duration or persistence of the photoadaptive response. For the future, it is worthwhile experimenting with a greater range of photoadapting doses, as well as challenge doses. In the present study, the photoadaptive response as a whole, lasted a period of 12 weeks; it would be worthwhile continuing the study for a longer period, with progressively smaller challenge doses. It would also be valuable for epidermal thickness to be re-measured at a time point when the photoadaptive response is thought to have normalized. It is of interest to observe how long it takes for epidermal thickness to return to normal.

Overall, this study has been valuable in shedding light on the human photoadaptive response and what constitutes photoadaptation. We have come to the conclusion that an increase in pigmentation is imperative in protecting the epidermis from subsequent UV injury. Thickening of the epidermis has also been shown to be another mechanism by which the epidermis safeguards itself from further UV injury. This study has elucidated the time frame of the photoadaptive response as best it can. Of interest is how the photoadaptive response differs in individuals of different skin types. Both mechanisms, hyperpigmentation and epidermal thickening, are shown to have been activated at different levels, and to different degrees, depending one's racial skin type. The various methods that have been used in this study have been unique and innovative, yet reproducible and reliable. It is hoped that this thesis will enlighten and provide direction for future investigators on the topic of the photoadaptive response of human skin.

## **ACKNOWLEDGEMENTS**

I would like to acknowledge the contribution of friends and colleagues that has made the work within this thesis possible.

- Dr Alison Hennessy performed the UV Transmission Spectrophotometry of the harvested epidermal samples. Using the Fluoramax software and the mathematical model described in Chapter 4, she then derived the relative thickness and melanin content of the samples. This is relevant for Chapters 4 and 5.
- Professor Brian Diffey was instrumental in developing the mathematical model which allowed for indirect calculations of epidermal thickness. This is relevant for Chapters 4 and 5
- Ms Yvonne Bisset performed the genotyping for MC1R polymorphism. This is relevant for Chapters 2 & 3.
- Dr Kazumasa Wakamatsu in Japan was extremely helpful in running the chemical assays for determining eumelanin and pheomelanin concentrations of freeze-dried epidermal samples. This is relevant for Chapter 4 and 5.
- Professor Jonathan Rees, with his tireless effort and constructive advice, was paramount in providing the vision and direction for this work to have been possible in the first place.
- The funding for this work was made possible by the Wellcome Trust.

Work undertaken by myself in study chapters (2-5) included:

- Development of the noradrenaline iontophoresis technique
- Design of the four studies described in Chapters 2, 3, 4 and 5.
- Design of questionnaires, information sheets, consent forms and recruitment of subjects.
- Repeated irradiation of subjects with all UVB sources
- Repeated measurement of doppler flux / erythema
- Repeated measurement of pigmentation
- Suction blistering and harvesting of epidermal samples
- Freeze drying of epidermal samples
- Processing and interpretation of data for the work above

## **DECLARATION**

I declare that the work presented in this thesis is fully original.

Dr Carol Chiu Yen Oh Adib



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## **APPENDIX**



Professor Jonathan Rees FmedSci  
Dermatology  
Lauriston Building, Royal Infirmary  
Lauriston Place  
EDINBURGH EH3 9HA  
☎ 44 (0)131 536 2041  
✉ 44 (0)131 2298769  
✉ [jonathan.rees@ed.ac.uk](mailto:jonathan.rees@ed.ac.uk)

[www.derm.med.ed.ac.uk/staff/rees.htm](http://www.derm.med.ed.ac.uk/staff/rees.htm)

## **PATIENT/VOLUNTEER INFORMATION SHEET**

### **Quantitative Physiology of Human Pigmentation**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others as you wish. Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

#### The purpose of the study.

Understanding why people's skin differs in response to ultraviolet radiation is important clinically. People who are very sun sensitive are more likely to burn in the sun, they are more likely to burn in response to treatments used by dermatologists and they are at an increased risk of skin cancer. We wish to understand the relation between the genes people inherit and the sensitivity of their skin to ultraviolet radiation. The purpose of the present study is to relate changes in genes to how people differ in response to irradiation (sunlight) on more than one occasion, and relate these differences to differences in skin colour and differences in hair colour.

*What groups of people are we asking whether they would take part in the study?*

We are particularly interested in people with red hair and for comparison people without red hair. Because hair changes with age we also would favour using people between the ages of 18-30 although strict cut off's are not critical.

The sort of research we are carrying out is non-therapeutic. Although we hope that it will increase our understanding of science relevant to medicine, people taking part in the study can expect no direct benefit.

We are asking people whether they would be willing to volunteer for these studies. Such volunteers can however include people who may be attending the hospital for other reasons.

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you are a patient attending the Infirmary whether you agree to take part, or whether you agree to take part and then subsequently change your mind and decline, will not affect the standard of treatment you receive in any way now or in the future.

### What will happen to me if I take part?

We are asking individuals whether they may be able to help with the study in a number of ways: because there are many options within this research study we have numbered the different procedures.

1. Allow us to measure their skin colour and hair colour using a modified camera and to provide a 10ml sample of blood and a small sample of their hair (equivalent to the amount of hair in an eyebrow but taken from the scalp).
2. Allow an area of skin, on the back, approximately 9 x 9cm to be exposed to ultraviolet radiation to a maximum of 10 occasions. The same area may be exposed up to a maximum of 10 occasions but most often, only 5 occasions of exposure will be required. Such irradiations will take place at daily to weekly intervals.
3. Individual irradiations take about 30 seconds each and the reading of the degree of redness about 1 minute per sample. The total procedure on each occasion may therefore take around 15 to 20 minutes.
4. Such areas of irradiation will cause redness and subsequently will cause the skin to pigment (or tan). The pigment or tanning may last in some people up to approximately four months. Such tests do not damage the skin permanently. The aim of our irradiation is to make the skin go slightly pink but not bright red. They are not generally painful.
5. Take samples from the superficial part of the skin. This technique is called "suction blistering" and is explained below.
6. In order to measure skin colour we wish to blanch the skin (get rid of the redness due to the blood flow). We do this by a technique called "iontophoresis" which involves passing a small electric current from a battery through the skin and a drug applied to the skin surface. This is explained in more detail below.

### Suction blisters

We wish to take superficial areas of skin to allow us to study the change in pigment between different people. The way we perform this is by raising up to 5 small blisters on the skin using a suction blister machine. This applies a negative pressure to the skin for approximately 45 minutes to 1 hour which induces small blisters measuring 0.4 to 0.5 of a centimetre across. The blisters can then be removed with a sterile blade and a dressing applied to minimise any resulting pain. Because only the superficial area of skin is removed, the skin grows back like the way skin grows back following a graze. Coverage of the area that has been blistered takes approximately 10 days, and during this time, like a graze, there may be intermittent discomfort. The area of skin will remain browner than the surrounding area of skin for somewhere between 2 and 8 weeks. Such a procedure does not scar the skin, i.e. it usually produces no lasting effects.

### Iontophoresis of Noradrenaline.

We wish to measure both how brown the skin goes in response to irradiation and also how red it goes. To do this properly, we need to temporarily reduce the blood flow to allow the brownness of the skin to be measured. We do this by passage of a small electric current (from the sort of battery that you would use in a radio - not mains electricity) to pass a drug, Noradrenaline, through the skin which then blanches that area of skin. Iontophoresis is a safe technique, uses minimal amounts of electricity but may be felt by some individuals as a slight prickling sensation for the duration of the iontophoresis, which may take up to 20 minutes. Some people are aware of this prickling sensation whereas others do not appear to feel it. Such iontophoresis would be applied to the areas of skin that had been repeatedly irradiated on the back or the buttock.

### Iontophoresis

Iontophoresis would be carried out on a maximum of 9 occasions.

### The possible benefits of taking part

It is important to emphasise as stated earlier that there are no direct benefits of taking part in this research for you. You should also understand that there is some minor discomfort involved such as the pain from the taking of blood, the prickling sensation as a result of iontophoresis and discomfort after the taking of blisters. In addition, we appreciate, that depending on which procedures you consent to, we are calling upon your time but in significant degree.

### What if something goes wrong?

Before conducting any research on humans considerable thought is given to likely benefits and disadvantages.

We feel there are no significant risks to you if you agree to take part in this study. In the unlikely event that you needed urgent advice during the course of the study or afterwards please contact Professor Jonathan Rees on telephone number 0131 536 2041 or the on call Dermatology registrar via the Royal Infirmary switchboard telephone number 0131 536 1000. Alternatively, if you wish to talk to an independent doctor then Dr Roger Aldridge (Patient Services Director for Dermatology, 1<sup>st</sup> Floor, The Lauriston Building, Lauriston Place, Edinburgh, EH3 9YW, telephone 0131 536 2067) would be happy to provide more information.

None of the doctors involved in this study have any financial interests in the research being carried out, nor are they being paid for organising this research. This research is being funded by The Wellcome Trust, a medical research charity through grants awarded to The University of Edinburgh.

All information which is collected about you during the course of the research shall be kept strictly confidential. Any information about you which leaves the Hospital will have your name and address removed so that you cannot be recognised from it.

The results of the research study will be published in medical journals available in the public domain.

It is our normal practice, with the patient's consent, to let their GP know that they have taken part in a research study. We will therefore write to your GP saying that you are partaking in the study unless you wish us not to.

### Contact for Further Information

#### **Dr. Carol Oh**

Wellcome Trust Clinical Research Fellow

Room 4126  
Department of Dermatology  
1<sup>st</sup> Floor Lauriston Building  
Lauriston Place  
Royal Infirmary of Edinburgh  
EH3 9HA

Tel: 0131 536 6091

Email: [carol.oh@ed.ac.uk](mailto:carol.oh@ed.ac.uk)



Dr. Carol Oh  
Clinical Research Fellow  
Department of Dermatology  
1<sup>st</sup> Floor, Lauriston Building  
Lauriston Place  
Edinburgh  
EH3 9HA  
Tel: +44-131-536 6091  
Email: [carol.oh@ed.ac.uk](mailto:carol.oh@ed.ac.uk)

**Questionnaire – Human Pigmentation Study**

NAME	HOME PHONE
ADDRESS	WORK PHONE
	MOBILE PHONE
	EMAIL
DATE OF BIRTH	GP NAME/ADDRESS/PHONE
MALE/FEMALE	
OCCUPATION	
ETHNIC ORIGIN	
EYE COLOUR	

---

Please Circle Your Response

- |  |          |
|--|----------|
| 1. Are you pregnant?   | YES / NO |
| 2. Are you receiving PUVA treatment?   | YES / NO |
| 3. Have you received over 500J of PUVA in the past?  | YES / NO |
| 4. Do you use a sunbed?  | YES / NO |
| 5. Do you take any medication that react with light?<br>(eg. minocycline, tetracyclines, thiazide diuretics/water tablets,<br>amiodarone, chlorpromazine, ciprofloxacin) | YES / NO |
| 6. Do you suffer from a mental illness or dementia?  | YES / NO |
| 7. Do you take antidepressants (MAOI/TCA) ?  | YES / NO |

Medication name: \_\_\_\_\_



8. **Do you take medication for heart problems or blood pressure?**(eg. beta-blocker)

YES / NO

**Medication name:** \_\_\_\_\_

9. **Do you have a pacemaker?**

YES / NO

10. Do you suffer from angina (chest pain)?

YES / NO

11. Have you suffered a heart attack in the past year?

YES / NO

12. Do you suffer from blood clots (thrombosis)?

YES / NO

When was most recent? \_\_\_\_\_

13. Do you suffer from lung problems ( emphysema/chronic bronchitis)?

YES / NO

---

List all the MEDICAL CONDITIONS you have had in the past and at present (including operations).

---

List all MEDICATION you are taking.

---

List all your ALLERGIES.

- 
14. Do you have a history of any skin condition?

YES / NO

(eg. eczema/psoriasis/skin cancer)

15. If you are undergoing treatment for this, please specify treatment, and when you last had the treatment \_\_\_\_\_

16. Have you ever received phototherapy/light treatment? YES / NO  
If so, when did it finish? \_\_\_\_\_
17. Do any other members of the family have a skin condition? YES / NO
18. Are you a twin? YES / NO
19. Which best describes the way your skin responds to sunlight?  
a) always goes red (sunburn), never tans  
b) goes red usually, sometimes tans  
c) sometimes goes red, but usually tans  
d) never goes red, always tans
20. When did you last have a sunny holiday abroad? \_\_\_\_\_
21. In natural British sunlight, how long does it take for your skin to go red?  
\_\_\_\_\_
22. In natural sunlight abroad, how long does it take for you skin to go red?  
\_\_\_\_\_
23. In natural British sunlight, how long does it take for your skin to tan?  
\_\_\_\_\_
24. In natural sunlight abroad, how long does it take for your skin to tan?  
\_\_\_\_\_
25. Have you ever been sunburnt? YES / NO  
How many times \_\_\_\_\_
26. Have you ever blistered as a result of sunburn? YES / NO
27. Do you have/get freckles? YES / NO
28. Do you smoke? YES / NO  
How many per day \_\_\_\_\_
29. Are you on any special diet? (vegetarian, low salt, diabetic, etc) YES / NO
30. How much alcohol do you consume in a week? \_\_\_\_\_
31. Females: when was your last menstrual period? \_\_\_\_\_
-



Professor Jonathan Rees FmedSci  
Dermatology  
Lauriston Building, Royal Infirmary  
Lauriston Place  
EDINBURGH EH3 9HA  
☎ 44 (0)131 536 2041  
✉ 44 (0)131 2298769  
✉ [jonathan.rees@ed.ac.uk](mailto:jonathan.rees@ed.ac.uk)  
[www.derm.med.ed.ac.uk/staff/rees.htm](http://www.derm.med.ed.ac.uk/staff/rees.htm)

## CONSENT FORM FOR ADULTS

### Quantitative Physiology of Human Pigmentation

**Further information is available from:** Professor Jonathan Rees, Grant Professor of Dermatology, 1<sup>st</sup> Floor Lauriston Buildings, Lauriston Place, Edinburgh. Telephone 0131 536 2041. E-mail: [jonathan.rees@ed.ac.uk](mailto:jonathan.rees@ed.ac.uk).

### List any drugs and procedures to be given in the study explaining their action:

1. Measuring skin and hair colour
2. Sample of venous blood.
3. Exposure to graded doses of ultraviolet radiation on one up to a maximum of 10 occasions affecting an area of skin approximately 9 x 9cm.
4. Raising of suction blisters on the arm, lower back or buttock (1 area only).
5. Use of Noradrenaline iontophoresis (passage of a small electric current from a battery) using the drug Noradrenaline to make the skin blanch to areas that have gone red from ultraviolet radiation exposure (maximum of 9 occasions).

- I agree to participate in the study parts 1, 2, 3, 4, 5.  
(Please circle parts you are happy to take part in).
- I have read this consent form and in the information sheet and had the opportunity to ask questions about them.
- I agree for notice to be sent to my General Practitioner.
- I agree to the provision of any clinically significant information to be given to my General Practitioner.
- I understand that I am under no obligation to take part in this study and that a decision not to participate will not alter any future treatment that I would receive or any treatment I am currently receiving.
- I understand that I have the right to withdraw from this study at any stage without giving a reason and again that this would not influence any treatment I am currently receiving or any treatment I might receive at some future date.
- I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

Signature of Patient:.....

Signature of Investigator .....

Name of Patient .....

Name of Investigator : .....

Date: .....

Date: .....

## **Publications**

**The time course of photoadaptation and pigmentation studied using a novel method to distinguish pigmentation from erythema.**

Oh C, Hennessy A, Ha T, Bisset Y, Diffey B, Rees JL.  
J Invest Dermatol 2004;123(5):965-972

**The photoadaptive response to ultraviolet exposure in human skin using ultraviolet spectrophotometry.**

Hennessy A, Oh C, Rees J, Diffey B.  
Phodermatol Photoimmunol Photomed 2005:229-233

**Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation.**

Hennessy A, Oh C, Diffey B, Wakamatsu K, Ito S, Rees J.  
Pig Cell Res 2005;18:220-223

**Ultraviolet radiation sensitivity in vitiligo and adjacent normal skin.**

Oh C, Hennessy A, Rees J.  
Acta Derm Venereol 2006;86(4):380

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# The Time Course of Photoadaptation and Pigmentation Studied Using a Novel Method to Distinguish Pigmentation from Erythema

Carol Oh,\* Alison Hennessy,\* Tom Ha,\*<sup>1</sup> Yvonne Bisset,\* Brian Diffey,† and Jonathan L Rees\*

\*Systems Group, Dermatology, University of Edinburgh, Edinburgh, UK; †Medical Physics, University of Newcastle upon Tyne, Newcastle, UK

The dynamics of human pigmentation in response to ultraviolet radiation (UVR) remain poorly characterized. In part, this is attributable to methodological issues relating to the overlap in spectra of hemoglobin and melanin. We describe a new method, based on the recording of reflectance properties following iontophoresis of a potent vasoconstrictor, noradrenaline. This removes the influence of blood, allowing measurement of pigmentation, represented as  $L^*$  on the  $L^*a^*b^*$  scale. Blood flow was separately assessed using laser Doppler flowmetry. We show that there is a clear dose response with the dose of UVR administered, that pigmentation peaks at 1 wk and declines over the following 10 wk, but does not return to baseline within this period. We show clear differences in the degree, but not the temporal pattern of pigmentation between different pigmentary groups. We also report that the relation between facultative pigment and constitutive pigment is incomplete, with a wide scatter of responses for the development of pigmentation irrespective of constitutive levels. For comparison we also document overall photoadaptation and relate changes in pigmentation to the overall changes in photoadaptation.

Key words: iontophoresis/melanocortin-1-receptor (MC1R)/photoadaptation/pigmentation/tanning/ultraviolet radiation

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Exposure of skin to ultraviolet radiation (UVR) promotes an acute inflammatory response clinically characterized by erythema (Farr and Diffey, 1984b), and at the molecular level by a large number of biochemical changes and alterations in gene expression, notably the appearance of DNA damage with subsequent incomplete repair (Kulms and Schwarz, 2002a; Ichihashi *et al*, 2003). The action spectrum for erythema mirrors that of DNA damage (Young *et al*, 1998). Alongside these acute responses, a number of changes occur that are adaptive, in the sense that they result in a diminished future response to equivalent doses of radiation. This response, described as photoadaptation, is poorly understood, but is viewed as comprising at least two processes: facultative pigmentation and non-pigmentary photoadaptation (Soffen and Blum, 1961; Rosario *et al*, 1979b; Sterenborg and van der Leun, 1988c; de Winter *et al*, 2001a). The former of these is, as the name implies, a result of increased melanogenesis. Since melanin is photoprotective, the argument runs that any increase in melanin will result in a greater degree of photoprotection. Non-pigmentary photoprotection is even less well understood

mechanistically but, since photoprotection occurs in the absence of pigment (such as is seen in vitiligo) (Everett, 1961), then processes such as increase in the thickness of the stratum corneum and epidermis are thought to be causally important in the adaptive response (Pearse *et al*, 1987). Both pigment (melanin) and stratum corneum together with suprabasal keratinocytes contribute to the attenuation of UVR by the epidermis; the exact contribution of either in the resting state and in response to UVR is unclear.

Although a myriad of biochemical pathways have been identified that change in response to UVR (Campbell *et al*, 1993; Smith and Rees, 1994; Kulms and Schwarz, 2002b; Rees, 2002a, b, 2003), the factors that lead to differences between persons are only partially understood. Humans are strikingly polymorphic in the degree of constitutive pigmentation (Rees, 2003), and this undoubtedly explains much diversity in acute and chronic UVR responses. Globally, variation in skin melanin is the major factor explaining the differences in incidence rates of virtually all forms of skin cancer (Rees, 2002a, c). It is widely accepted that pigmentary status is, in general, genetically determined. With respect to non-pigmentary photoadaptation, beyond its existence, little is known (Soffen and Blum, 1961; Rosario *et al*, 1979a; Sterenborg and van der Leun, 1988b; de Winter *et al*, 2001b). To what degree it is under genetic control is unknown, nor whether it is entirely independent of pigmentary status (Rees, 2002b).

A major fillip to studies of human pigmentation has come from the identification of genes important in human melanin formation, notably the melanocortin-1-receptor (MC1R)

Abbreviations: MC1R, melanocortin-1-receptor; NA, noradrenaline; SED, standard erythema dose; SEM, standard error of the mean; UVB, ultraviolet B; UVR, ultraviolet radiation

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<sup>1</sup>Present address: Department of Dermatology, Addenbrookes Hospital, Cambridge, UK.



(Rees, 2003) and genes encoding tyrosinase and related proteins (Sturm *et al*, 2001). Work in model organisms, particularly the mouse, has identified a number of candidate genes, allelic variation which may underpin variation in human pigmentation. One rate-limiting issue remains, however, namely that our ability to describe and characterize human pigmentation at the physiological level remains poor (Rees, 2002b; Ha *et al*, 2003c). A number of methodological issues relating to the measurement of response to UVR have only recently been addressed. Examples include the site specificity of the acute inflammatory response<sup>2</sup> and the dose dependency of the photoprotective effects of melanin pigmentation between persons (Ha *et al*, 2003a). The methods of assessment in experimental studies have also often been weak, using endpoints such as the minimal erythema dose or the Fitzpatrick skintype classification, methods that although (arguably) suitable in the clinical context, are too imprecise for meaningful studies of human genetic diversity (Farr and Diffey, 1984a; Rampen *et al*, 1988; Westerhof *et al*, 1990; Wee *et al*, 1997; Rees, 2002b). One particular important methodological issue is the subject of this paper: how can the dynamic pigmentary response to UVR be objectively measured (in man) in such a way that the procedure is not too invasive so that a large number of data points can be obtained.

The response to a single exposure of UVR can easily and reliably be quantified objectively using reflectance techniques or by measures of blood flux using Doppler flowmetry (Farr and Diffey, 1984c; Farr and Diffey, 1985; Andersen *et al*, 1991). Similarly, reflectance instruments can be used to measure skin pigmentation (Park *et al*, 1999, 2002). Once the skin is exposed to UVR and erythema develops, however, it is not possible to easily measure changes in either blood flow or pigmentation as the spectrum of hemoglobin and melanin overlap. Because blood flow can remain elevated for several weeks after UVR exposure, this is a major drawback to reflectance techniques if they are used to measure pigmentation (Wagner *et al*, 2002a; Ha *et al*, 2003e). One attempt to circumvent this issue relies on use of measurements beyond 650 nm, the region where hemoglobin contributes little (Kollias and Baqer, 1985; Wagner *et al*, 2002b). The drawback of such an approach is that melanin absorption is also less in this region, and the technique relies on a linear extrapolation from results at a longer wavelength.

In this paper, we present a new method that we believe complements present methodology, and allows the dynamics of pigmentation to be objectively measured: erythema is measured using Doppler flux and pigmentation is measured by reflectance, but following iontophoresis of noradrenaline (NA) to induce vasoconstriction. We show that it is possible to define a dose response for pigmentation, describe the kinetics of pigmentation over several months, and demonstrate how change in pigmentation can be related to MC1R genotype and constitutive skin color.

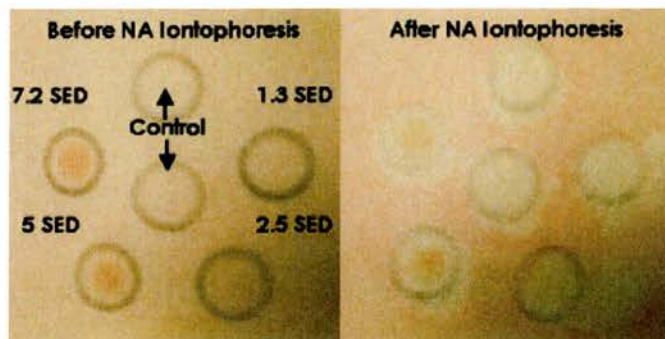
## Results

### The time course of pigmentation

**Suppression of erythema by NA iontophoresis** NA was successful in suppressing UV-induced erythema. Figure 1A shows irradiated skin areas before NA iontophoresis and Fig 1B shows the suppression of erythema of the irradiated spots following iontophoresis with NA.

The erythematous response was first examined prior to NA iontophoresis. Graphs in Fig S1 (supplemental figure for online viewing) measure the increase in flux of the irradiated areas, that is, how much more flux there is in each irradiated area over baseline unirradiated skin. In the *short-time course study*, UV-induced erythema measured by contact laser Doppler was highest on the first day following UVR (Fig S1A). There was an obvious dose response with the highest flux being generated by 7.2 standard erythematous dose (SED). The flux then fell dramatically over the first week. The differences in flux increase between Days 1 and 4 for the doses 7.2, 5, 2.5, and 1.3 SED were, respectively, 385 (standard error of the mean (SEM) 82) and 102 (SEM 55) ( $p = 0.003$ ), 299 (SEM 90) and 85 (SEM 53) ( $p = 0.006$ ), 81 (SEM 28) and 21 (SEM 10) ( $p = 0.1$ ), and finally -3 (SEM 4) and 1 (SEM 2) ( $p = 0.3$ ) (all paired  $t$  tests). In the *long-time course study* from 1 to 9–12 wk, an erythematous dose response was again obvious (Fig S1B). Over this time course, erythema was highest at Week 1, and then diminished thereafter.

The effect of NA iontophoresis on skin blood flux was then examined. It is worthwhile noting that normal unirradiated skin flux on the lower back varies from one individual to another, being as high as 70 absolute flux units (data not shown). Skin that appears blanched or vasoconstricted as a result of NA iontophoresis such as in Fig 1B, registers flux levels of 3–40 Doppler flux units. Following NA iontophoresis, flux of the irradiated spots measured by contact laser Doppler decreased substantially (Fig S1C, D). On Day 1, however, UV-induced erythema was not completely suppressible by NA iontophoresis and therefore color readings were not taken on Day 1 as the influence of change in blood flow could not be excluded. By Days 4 and 7, the



**Figure 1**  
**Suppression of ultraviolet (UV)-induced erythema by noradrenaline (NA) iontophoresis.** Photographs taken 1 wk post-UVR illustrate the difference in skin pigmentation color before and after iontophoresis with NA. Skin areas irradiated with UVB doses 1.3, 2.5, 5, and 7.2 standard erythematous dose (SED) as well as two control areas were iontophoresed with NA before pigment color measurements were taken.

<sup>2</sup>Waterston KM, Ha TK, Naysmith L, Weller R, Rees J: Photoadaptation is dose dependent, but not site dependent. *J Invest Dermatol* 121(1):1144, 2003 (abstr).



mean absolute flux following NA iontophoresis was 34 (SEM 11) and 26 (SEM 6), respectively. These were levels where the skin was blanched, and therefore pigment color readings were taken from Day 4 onwards. From then on, the technique of NA iontophoresis was successful in reducing flux to sufficiently low levels for the rest of the study period of 12 wk. Table S1 (supplemental table for online viewing) shows the changes in flux following NA iontophoresis throughout the study period from Day 1 to Week 12; it is worth noting the absolute flux levels of 30–40 U, values consistent with blanched skin, indicating successful suppression of erythema.

**Pigmentary dose response and time course** In examining the relationship between UVR dose and pigmentation, the L\* axis is of principal interest. A clear dose response is evident (Fig S2, available for viewing online) with higher doses of ultraviolet B (UVB) producing higher mean L\* values for any given time point. UVB radiation with 7.2 SED produced highest mean L\* values followed by radiation with 5 SED, 2.5 SED, and finally 1.3 SED.

The greatest change in L\* values was seen in the first week with the mean increase in L\* being higher on Day 7 than on Day 4 for all doses of UVB delivered (Fig S2A). The mean increase in L\* for Day 4 vs Day 7 for doses 7.2, 5, 2.5, and 1.3 SED was, respectively: 5 (SEM 0.5) vs 7 (SEM 1.1) (p = 0.04), 4 (SEM 0.6) vs 5 (SEM 0.9) (p = 0.09), 0.2 (SEM 0.5) vs 1.7 (SEM 0.9) (p = 0.3), and –0.8 (SEM 0.4) vs –0.5 (SEM 0.5) (p = 0.6). The difference in the mean increase in L\* between Days 4 and 7 for 7.2 SED was significant (p = 0.04). There was also a noticeable difference between the 2 d for the lower UVR doses although the difference was

not statistically significant. Should there have been an over-estimation of L\* on Day 4, as a result of incomplete suppression of erythema by NA iontophoresis then the “true” L\* on Day 4 would be lighter than represented; this potential error would have the effect of underestimating the finding that skin is darker on Day 7 than Day 4. Examination of L\* from 1 to 12 wk, shows that the mean increase in L\* for all doses was again highest at Week 1 (Fig S2B). The L\* value then decreased at Week 2. The mean increase in L\* for Weeks 1 and 2 for doses 7.2, 5, 2.5, and 1.3 SED was: 8 (SEM 0.8) and 3 (SEM 0.7) (p < 0.0001), 6 (SEM 0.7) and 3 (0.7) (p = 0.0001), 3 (SEM 0.4) and 1 (SEM 0.5) (p = 0.0067), and 0.6 (SEM 0.2) and 0.6 (SEM 0.5) (p = 0.9). The difference in L\* between Weeks 1 and 2 is statistically significant for doses 7.2, 5, and 2.5 SED. Therefore, over the entire study period from Day 4 to Week 12, L\* was maximal at 7 d (Week 1).

The temporal pattern of a\* values mirrored those of the L\* values, with a\* values being higher for all doses on Day 7 than on Day 4 (Fig S2C). Over the time course of 9–12 wk, a\* values were highest at Week 1 with 7.2, 5, 2.5, and 1.3 SED producing a mean increase in a\* of 6 (SEM 0.7), 4 (SEM 0.6), 1.5 (SEM 0.3), and 0.05 (SEM 0.19), respectively (Fig S2D). After Week 1, a\* values fell quickly but continued to remain above control values, even toward the end of the study period. A dose response was demonstrated by the a\* values.

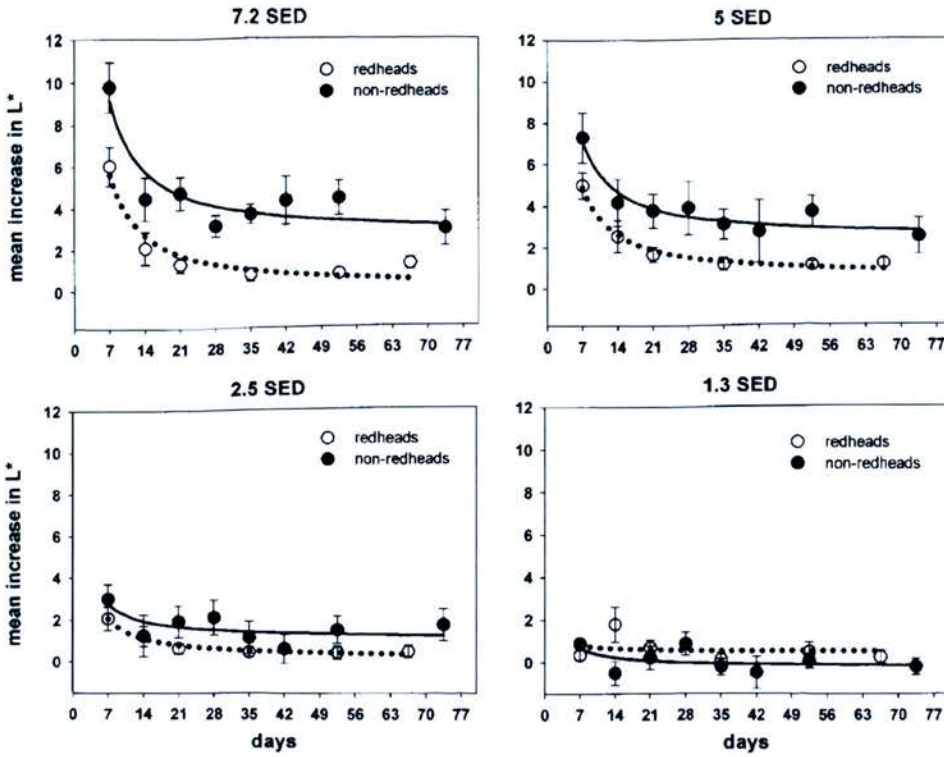
Similar to the L\* and a\* values, b\* values were higher on Day 7 than on Day 4 for the three higher doses of UVB (Fig S2E). Over the longer time course, however, b\* values did not show any obvious peak, unlike L\* and a\*; instead, there was a mild increase in b\*, which was sustained over Weeks 1 and 2 (Fig S2F). This response was seen for all doses except the lowest dose of 1.3 SED.

Table I. MC1R Genotype for subjects studied with and without red hair

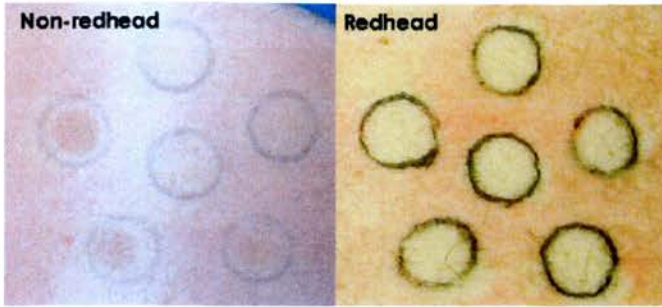
Redheads (n = 12)		Non-redheads (n = 12)	
Northern European	151 ht, 160 ht	Northern European	151 ht
Northern European	151 ht, 160 ht	Northern European	160 ht
Northern European	151 ht, 160 ht	Northern European	160 ht
Northern European	151 ht, 160 ht	Northern European	60 ht, 294 ht
Northern European	151 ht, 160 ht	Northern European	61 ht
Northern European	151 ht, 294 ht	Northern European	Consensus sequence
Northern European	151 ht, 294 ht	Northern European	Consensus sequence
Northern European	151 ht, 294 ht	Southern Asian	Consensus sequence
Northern European	151 ht, 294 ht	Middle Eastern	Consensus sequence
Northern European	151 ht, 294 ht	Middle Eastern	60 ht
Northern European	151 ht	Far East Asian	92 ht
Northern European	160 ht	Far East Asian	163 hm, 166 ht

ht, heterozygote; hm, homozygote.

**Groups with different pigmentary status** The 24 subjects who were followed over the 9–12 wk period comprised 12 redheads of Northern European descent (Group 1) and 12 non-redheads of varied ancestry including seven Northern European, one Southern Asian, two Middle Eastern, and two Far East Asian (Group 2) (Table I). Genotyping of the red hair group showed 10 of 12 to be homozygous and two heterozygous for known diminished function mutations of the MC1R (Schioth *et al*, 1999; Flanagan *et al*, 2000c; Ha *et al*, 2003b). In the non-redheaded group, no subject was homozygous, but several Europeans were heterozygote for diminished and highly penetrant MC1R loss of function alleles (Flanagan *et al*, 2000b). These results are compatible with previous population studies of MC1R diversity (Rana *et al*, 1999; Flanagan *et al*, 2000a, 2001a; Harding *et al*, 2000; Healy *et al*, 2000). Figure 2 shows the difference in response between Group 1 and Group 2 subjects. One week following UVB irradiation with 7.2 SED, Group 2 subjects showed an increase in L\* of 10 (SEM 1.2) whereas Group 1 showed an increase in L\* of only 6 (SEM 0.9) (p = 0.04). Likewise, the increases in L\* of Group 2 vs Group 1 for doses 5, 2.5, and 1.3 SED were 7 (SEM 1.2) vs 5 (SEM 0.6) (p = 0.05); 3 (SEM 0.7) vs 2 (SEM 0.6) (p = 0.06), and 1 (SEM 0.3) vs 0.3 (SEM 0.2) (p = 0.11). There was a significant difference between the two groups at 1 wk for the three higher doses; following 1.3 SED, however, the two



**Figure 2**  
**Difference between redheads and non-redheads.** The pigmentary response of redheads and non-redheads following various doses of ultraviolet B (UVB) analyzed with respect to the L\* value. Redheads (n = 12), non-redheads (n = 12). Vertical bars indicate  $\pm$  SEM. Polynomial inverse first-order curves were fitted using SigmaPlot 2002 Version 8.0 (SPSS Inc.).



**Figure 3**  
**Persistence of pigment color in redheads and non-redheads.** Photographs taken at 9 wk post-ultraviolet B (UVB) radiation illustrate the difference in retention of pigment color in redheads and non-redheads over this time course.

groups did not differ. The time course of change in L\* appeared to be the same for Group 1 and Group 2.

Figure 3 illustrates the persistence of pigmentation (post-NA iontophoresis) in both, a non-redhead and a redhead, at 9 wk post-UVB radiation. Pigmentation persisted for a greater length of time in Group 2 when compared with Group 1 (redheads).

**Relation between constitutive pigmentation and facultative pigmentation** In order to explore the relation between constitutive pigmentation and facultative pigmentation, the constitutive pigment L\* reading on the buttock was compared with the final L\* following the various irradiation doses on the back. These results are shown graphically in Fig 4. Almost all points lie below the line of identity ( $x = y$ ), signifying that following irradiation, almost every subject attained a darker pigment color (L\*). The degree of

scatter around the regression lines gives a measure of the correlation between constitutive and facultative L\*. R<sup>2</sup> values are 0.5, 0.7, 0.8, and 0.9 for 7.2, 5, 2.5, and 1.5 SED, respectively, indicating a decrease in correlation with increasing dose.

If the change in L\* is related to pigmentary status (Group 1 vs Group 2), only the highest dose (7.2 SED) shows any formally significant difference between groupings, with the difference in the means for the other groups being small (7.2 SED,  $p = 0.02$ ; 5 SED,  $p = 0.11$ ; 2.5 SED,  $p = 0.31$ ; 1.3 SED,  $p = 0.15$ ). The relation with skin type is also unremarkable (data not shown).

### The time course of photoadaptation

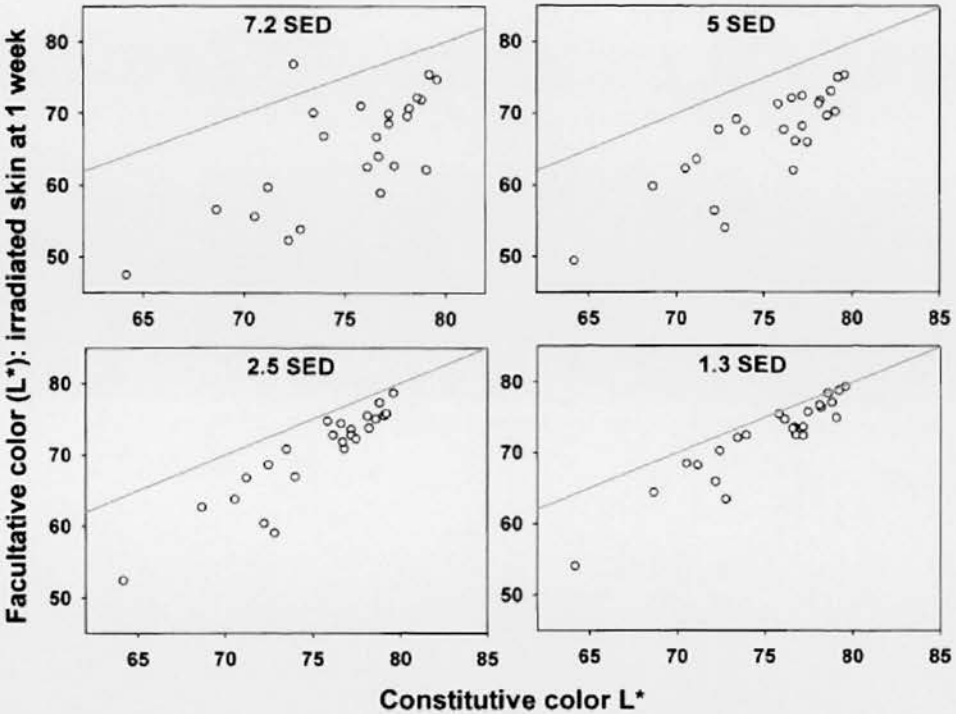
**Flux of adapted and unadapted skin following challenge** Following each challenge with UVB doses of 5.2, 8.2, and 13 SED, the mean increase in flux of the adapted site was compared with the mean increase in flux of the unadapted site. Figure 5 illustrates that on almost every occasion, the mean increase in flux of the adapted site was less than that of the unadapted site. This held true for Weeks 1/2, 1, 2, and 3–4, with a significant difference in mean flux increase between the adapted and unadapted sites ( $p < 0.05$ ). By Week 10, the mean flux increase of one site was not significantly different from that of the other following challenges with 8.2 and 13 SED; however, there remained a significant difference in mean flux increase following challenge with the lowest dose, 5.2 SED ( $p = 0.03$ ).

### Discussion

The principal purpose of this work was to introduce a method that would allow the study of the physiology of



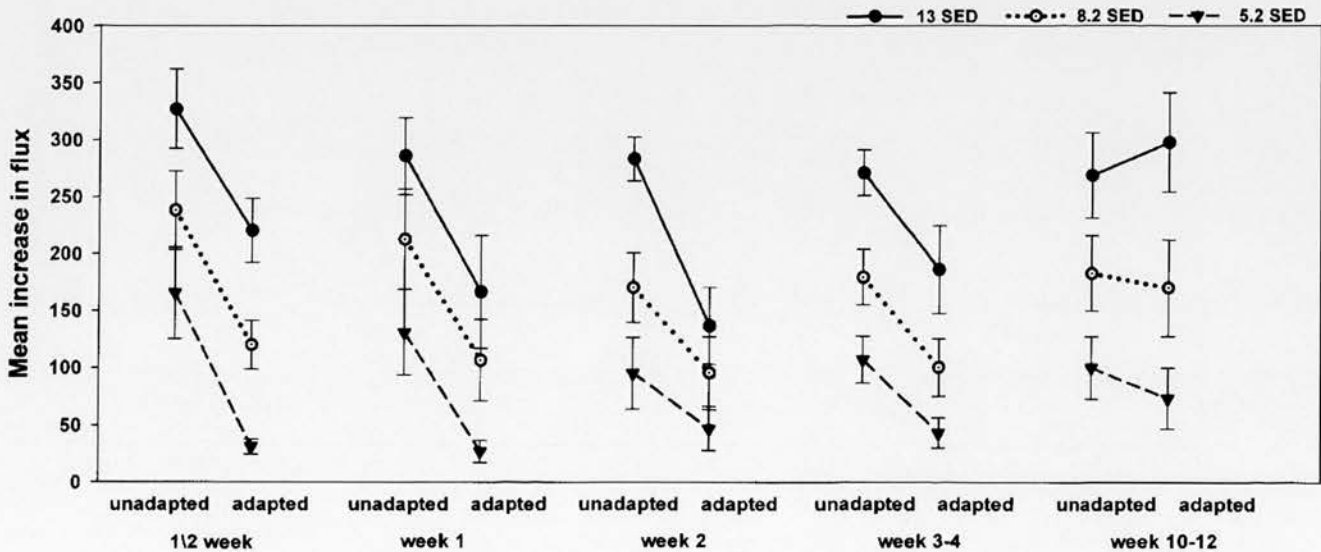
**Figure 4**  
**Comparing facultative L\* with constitutive L\*.** At 1 wk post-ultraviolet radiation (UVR), the pigment color of irradiated back skin for the various doses was compared with constitutive skin color. The continuous line is the line of identity signifying a hypothetical situation in which the constitutive L\* and facultative L\* are identical ( $x = y$ ). The dashed line is the regression line through the data points.



pigmentation in man in response to UVR. The need for such a measure is obvious. Currently, the overlap in spectra for hemoglobin and melanin means that methods based on reflectance are problematic (Anderson and Parrish, 1981; Kollias, 1995). Either, one uses  $a^*$  and  $L^*$  scores, and obtains data that are subject to systematic error, or alternatively you rely on linear extrapolation from readings  $>650\text{ nm}$  (Wagner *et al*, 2002c). Our approach was more direct: to use Doppler flowmetry to assess blood flow as a measure of vascular response (Farr and Diffey, 1984d), and color on the  $L^*$  or  $a^*$  scales as a measure of pigmentation when blood flow had been eliminated by pharmacological manipulation. Of course

our method, relying just on color to assess pigmentation, is in itself incomplete. When pigment is produced, its packaging, distribution within the epidermis and optical properties in the ultraviolet wavebands all may play an important part in its physiological function, subtleties that are beyond our assay system (Jimbow and Sugiyama, 1998). Conversely, however, the system we propose allows a quantitative measure to be obtained in man, without being too invasive, and allows serial measures on the same person.

We show that iontophoresis with NA can inhibit flux at all but the earliest stages following exposure to large doses of UVR. We show that pigment is greatest at day 7 and



**Figure 5**  
**Flux increase of unadapted and adapted skin following ultraviolet B (UVB) challenge.** UV-induced erythema, measured as flux, of photo-adapted skin was compared with that of unadapted skin following a challenge with three different doses of UVB. Vertical bars indicate  $\pm$  SEM.  $n = 11$ .

declines thereafter but is still present above basal levels 10 wk later. We show that we can produce a straightforward dose response of pigment with UVR dose, as expected, and that the time course of disappearance of pigmentation is the same at different challenge doses. We also validated our method, in two groups of subjects, expected to have different pigmentary characteristics, a group of Northern Europeans with red hair and a mixed group of Northern Europeans and Asians without red hair. As expected, we show differences in the development of facultative pigment between these groups but the likenesses are perhaps more worthy of comment. Both groups showed a dose response and the time course of disappearance of pigment was similar in both groups. To what extent people develop facultative pigmentation is a matter of degree, and although the means of these groups are different, the overlap is considerable.

We also related the degree of increase of pigment on the test sites to constitutive pigment on the buttock, by UVR dose. These results are intriguing in that we see little relation between constitutive pigment and facultative pigmentation. It is also worthy of comment that even when grouped by pigment type (Group 1 or Group 2) that little difference in the increase in pigment is seen except at the highest dose (7.2 SED). The explanation for this, we believe, is quite straightforward: people able to tan (such as one thinks of in Fitzpatrick Type IV skin) are not more able to increase their pigment than some with Type I skin per unit of UVR, what they are able to do is tolerate a bigger dose of UVR to begin with, that enables a greater stimulus to be made experimentally. In our experiment this was not the case, as fixed doses of UVR were administered to all subjects irrespective of constitutive color. In our data, we see little evidence for anything but a continuous distribution of abilities to tan, just as there is a distribution of values for constitutive pigmentation. Of note is that the highest exposure dose of UVR we gave corresponded to exposure to 1 h of midday sunshine at latitude 40° North (e.g. New York) on a day in July. Nonetheless, it is obvious from experience that a different ceiling applies to the degree of development of pigmentation; the relation between the rate of change of pigmentation and the maximum pigmentation that can be developed requires further work.

Pigmentation is one method of photoadaptation, but it is known that other processes contribute to the development of photoadaptation (Blum *et al*, 1959; Soffen and Blum, 1961; Rosario *et al*, 1979c; Sterenborg and van der Leun, 1988a; de Winter *et al*, 2001c). One such process is non-pigmentary photoadaptation; its mechanism(s) and characteristics, however, are little studied. In the present work we could not assay this process directly, merely overall photoadaptation, by the use of repeated irradiation and subsequent challenge. We have wondered whether the time course differed between these two measures, and if so, by implication, the time course of non-pigmentary photoadaptation was different. What we observed was that the time course of overall photoadaptation was similar to pigmentary change, although we would accept that the power of our design to detect differences may have been small. Further work on the relation between pigmentary and non-pigmentary photoadaptation is ongoing.

Finally, we would emphasize the importance of the study of the macroscopic development of pigmentation and photoadaptation. The relation between UVR and different types of skin cancer is well known (Setlow, 1974; Rees and Healy, 1997; Sturm, 2002). A clear role for the pattern of exposure is suggested for both melanoma and basal cell carcinoma (as compared with squamous carcinoma). Site differences within a person in tumor density and response to UVR are also evident.<sup>2</sup> It seems reasonable to imagine that some of these differences may be accounted for in terms of the dynamics of the photoadaptive response to UVR.

Macroscopic differences in pigmentary characteristics between people are, in general, genetically determined (Rees, 2003). We suggest that pigmentary differences between people should be treated as experimental variables in a population for a more detailed study.

## Materials and Methods

Two separate studies were performed: (1) the time course of pigmentation and (2) the time course of photoadaptation.

### The time course of pigmentation

**Subjects** Twenty-eight healthy volunteers (13 males, 15 females) were recruited from the Department of Dermatology, Royal Infirmary of Edinburgh. Group 1 comprised 12 redheads of pale constitutive skin color (four males, eight females); all were of Northern European ancestry. Group 2 comprised 16 non-redheads of relatively darker skin color (nine males, seven females); of this group, 11 were Northern European, two Middle Eastern, one Southern Asian, and two Far East Asian. These two groups were chosen on the basis of convenience and were designed to include a range of world skin types, excluding Africa. Redheads were recruited on visual inspection of hair color. Each of the subjects received four doses of UVB from a 300-W Xenon Arc Lamp (Oriol, Stratford, California) optically coupled to an interference filter (Andover 300FS10-50 AM-33230-01; peak transmittance 300 nm Oriol, Leatherhead, Surrey, UK) and traceable to national standards (Ha *et al*, 2003d). The UVR was delivered onto the right lower back in small spots of 1 cm in diameter. The spots were arranged in a pattern to accommodate two control unirradiated spots (Fig 1A). UVB administered, expressed in SED, was 1.3 SED (28 mJ per cm<sup>2</sup>), 2.5 SED (56 mJ per cm<sup>2</sup>), 5 SED (112 mJ per cm<sup>2</sup>), and 7.2 SED (160 mJ per cm<sup>2</sup>) (Diffey *et al*, 1997). The SED was chosen as it is the unit of erythral radiation (Harrison and Young, 2002); it requires an exposure of about 3 SED to produce just perceptible erythema in the unacclimatized white skin of the most common northern European skin types. An exposure of 5–8 SED will result in moderate sunburn and 10 SED or more can result in a painful, blistering sunburn. Following this single irradiation, the pigmentary color of these spots was measured over the subsequent 9–12 wk in two separate substudies: the short-time course study and the long-time course study. The short-time course study was carried out to further refine the results obtained in the long-time course study. In the long-time course study, pigment color of the irradiated spots of 24 subjects (12 males, 12 females) was measured up to eight times over a 9–12 wk period. In the short-time course study, pigment color of the irradiated spots of six subjects (four males, two females) was measured twice over a 1 wk period. Two subjects participated in both the short- and the long-time course studies.

The MC1R status of the subjects was determined by automated sequencing of DNA extracted from whole blood according to previously published methods (Flanagan *et al*, 2001b). All subjects gave informed consent prior to participating. Volunteers were recruited from a secondary care facility and all studies had appropriate ethics committee approval, and were made in the light of the



declaration of Helsinki (<http://www.wma.net/e/policy/b3.htm>) and subsequent amendments on the world medical association web site (<http://www.wma.net/e/>). Volunteers were not paid.

**NA iontophoresis** In order to eliminate the influence of erythema when taking measures of pigment color, iontophoresis with NA was employed (Drummond and Lipnicki, 2001). NA, when iontophoresed through skin, has the effect of producing a temporary vasoconstriction of dermal vasculature, potentially eliminating any erythema induced by UVR, allowing the "true" pigment color (i.e. that because of melanin) to be measured. The positive electrode of the iontophoresis machine (Phoresor II PM-850, Iomed, Salt Lake City, Utah) was clipped to a metal plate, under which were arranged cotton wool pads (1 cm diameter, 0.2 cm thick) soaked with NA (1:1000, Abbott Laboratories Ltd, Kent, UK). These pads were strategically placed over the four irradiated spots and two control unirradiated spots. The metal plate was then taped to the skin of the subject and a 9 V battery-sourced direct current of 0.5 mA was run over a 5–10 min period. Underlying skin blood flux was measured in triplicate using a contact laser Doppler probe (Moor Instruments, Devon, UK) before and after iontophoresis, to ensure satisfactorily low flux prior to measuring pigment color.

**Colorimetry** Once the irradiated areas were sufficiently blanched and free of erythema (assessed using the contact laser Doppler), the color of the irradiated spots was measured using the Minolta Chromameter CR300 (Osaka, Japan). Color readings were represented on three dimensions using the  $L^*a^*b^*$  system (Commission Internationale de l'Eclairage, <http://www.cie.co.at/cie/>).  $L^*$  represents the color scale from black to white (0–100),  $a^*$  represents the scale from green to red (–60 to 60), and  $b^*$  represents the scale from blue to yellow (–60 to 60). Readings were taken in triplicate for each of the six spots (four irradiated and two controls). This procedure of NA iontophoresis followed by colorimetry was performed at each time point of measurement over the 9–12 wk period for each subject.

### The time course of photoadaptation

**Adaptation phase** Eleven healthy subjects were recruited (two males, nine females). In order to promote a photoadaptive response, they were each delivered three doses of UVB to a square measuring 9 cm × 9 cm on the right lower back over 3 consecutive days (one dose per day). The UVB source was the Waldmann UV 801 TL-01 (Herbert Waldmann GmbH & Co. KG, Villingen-Schwenningen, Germany). Doses delivered were: 2.7 SED (480 mJ per cm<sup>2</sup>), 2.9 SED (520 mJ per cm<sup>2</sup>), and 3.2 SED (570 mJ per cm<sup>2</sup>) and were similar to the doses used routinely for the treatment of psoriasis. The left lower back was not irradiated and therefore served as an unadapted control.

**Challenge phase** At the end of the photoadaptation phase, each subject was challenged with three higher doses of UVB, 5.2 SED (189 mJ per cm<sup>2</sup>), 8.2 SED (300 mJ per cm<sup>2</sup>), and 13 SED (476 mJ per cm<sup>2</sup>), on each occasion over the next 10–12 wk, delivered as small spots of 0.8 cm in diameter using a TL-12 UVB machine engineered by Professor Brian Diffey (Regional Medical Physics, University of Newcastle, UK). This procedure involved administration of these three doses onto the previously exposed square (right lower back), and also onto the unadapted area of skin as a control (left lower back). Photoadaptation was defined by the difference in erythema response between the adapted and unadapted areas following the standard challenge doses. The challenges were administered at several time points following the last day of the photoadaptation phase; 1/2, 1, 2, 3–4, and 10–12 wk.

**Measurement of erythema and pigment color** Erythema was measured 24 h following each challenge (at 1/2, 1, 2, 3–4, and 10–12 wk) using a contact laser Doppler instrument (Moor Instruments). The

challenge-induced flux readings on the adapted area were then compared with those of the unadapted area.

**Graphical and statistical analysis** Graphical analysis was performed using SigmaPlot 2002 Version 8.0 (SPSS Inc., Chicago, Illinois) and Microsoft Excel 2000 (Microsoft, Seattle, Washington). StatsDirect Version 2.3.2 (StatsDirect Ltd, Sale, Cheshire, UK, <http://www.statsdirect.com/>) was used for statistical analysis. Levels of significance were calculated using paired *t* tests.

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### Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23464/JID23464sm.htm>

**Figure S1. Increase in flux of UV-irradiated skin before and after NA iontophoresis.** Flux of skin areas irradiated with 4 different doses of UVB was measured with a contact laser Doppler instrument before (A, B) and after (C, D) NA iontophoresis from Day 1 to Week 12. The flux of control unirradiated skin was also measured and then subtracted from that of the irradiated areas, to derive the increase in flux above baseline of these irradiated areas. Figs 2A and C, *n* = 6; 2B and 2D, *n* = 24. Vertical bars represent ± SEM. Curves in 2B are exponential decay curves obtained using SigmaPlot 2002 Version 8.0 (SPSS Inc., Chicago, USA).

**Figure S2. Mean increase in  $L^*a^*b^*$  values following a single event of UVB radiation.** Following a single irradiation with various doses of UVB, pigment color measurements were taken using the (A, B)  $L^*$  value, (C, D)  $a^*$  value and (E, F)  $b^*$  value after iontophoresis with NA. Figs 3A, C and E, *n* = 6; Figs 3B, D and F, *n* = 24. Vertical bars represent ± SEM. In Figs 2B and D, polynomial inverse 1<sup>st</sup> order curves were fitted using SigmaPlot 2002 Version 8.0 (SPSS Inc., Chicago, USA).

**Table S1.** Changes in flux before and after noradrenaline iontophoresis

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Address correspondence to: Professor Jonathan L Rees, Department of Dermatology, Room 4018, 1st Floor Lauriston Building, Lauriston Place, Edinburgh EH3 9HA, UK. Email: [jrees@staffmail.ed.ac.uk](mailto:jrees@staffmail.ed.ac.uk)

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## The photoadaptive response to ultraviolet exposure in human skin using ultraviolet spectrophotometry

Alison Hennessy<sup>1</sup>, Carol Oh<sup>1</sup>, Jonathan Rees<sup>1</sup>, Brian Diffey<sup>2</sup>

<sup>1</sup>Department of Dermatology, University of Edinburgh, Edinburgh EH3 9HA, UK and <sup>2</sup>Regional Medical Physics Department, Newcastle General Hospital, Newcastle NE4 6BE, UK

**Background:** Both pigmentation and non-pigmentary processes contribute to the development of photoadaptation yet the exact contribution of either in the resting state and in response to ultraviolet (UV) radiation is unclear. The purpose of this study was to estimate independently these changes occurring in the epidermis following repeated exposure to UV in two groups with differing degrees of constitutive pigmentation.

**Methods:** We describe a mathematical model for explaining the spectral absorbance of excised human epidermis based on the absorbance of constituent chromophores. The model was applied to spectral absorbance data measured on samples of epidermis excised from pre-irradiated skin and from skin obtained following UV irradiation on 3 successive days.

**Results:** We found that in Asian skin there was only a mild photoadaptive response, principally by a small increase in pigmentation. On the other hand, the significant adaptive response in Caucasian skin was through hyperplasia of the epidermis, with tanning contributing only to a much smaller degree.

**Conclusion:** This study has enabled us to study independently the pigmentary and non-pigmentary pathways and has shown that in those people with a lower degree of constitutive pigment, the primary mechanism of photoadaptation is via the non-pigmentary route.

**Key words:** absorption; epidermis; hyperplasia; melanin; photoadaptation.

Exposure of skin to ultraviolet (UV) radiation promotes an acute inflammatory response clinically characterized by erythema. Alongside this acute response, a number of changes occur that are adaptive, in the sense that they result in a diminished future response to equivalent doses of radiation. This response, described as photoadaptation, is poorly understood, but is viewed as comprising at least two processes: facultative pigmentation and non-pigmentary photoadaptation (1–4). The former of these is a result of increased melanogenesis.

Since melanin is photoprotective, it follows that any increase in melanin will result in a greater degree of photoprotection. Non-pigmentary photoprotection is less well understood mechanistically, but as photoprotection occurs in the absence of pigment, such as is seen in vitiligo (5), processes such as increase in the thickness of the epidermis are thought to be causally important in the adaptive response (6). Both pigment (melanin) and stratum corneum together with suprabasal keratinocytes contribute to the attenuation of

UV radiation by the epidermis; the exact contribution of either in the resting state and in response to UV radiation is unclear.

The purpose of this study was to estimate the hyperplasia and pigmentary changes occurring in the epidermis following repeated exposure to UV radiation in two groups with differing degrees of constitutive pigmentation. Given the difficulty with making an absolute measurement of excised epidermal thickness because of problems such as changes in hydration and shrinkage (7), we have developed a method for estimating the changes in epidermal thickness and pigmentation that occur following UV irradiation and that makes measurement of skin thickness unnecessary.

### Materials and methods

#### Subjects

Twenty healthy volunteers (four Asian and 16 Caucasian) and eight subjects with vitiligo were recruited from the Edinburgh area. All subjects gave their



informed consent prior to participating. The study had the appropriate Ethics Committee approval (LREC/2003/4/2).

#### UV irradiation and spectrophotometry

The 20 healthy volunteers received three exposures to UV radiation from a Philips type TL-01 lamp (Philips Lighting, Eindhoven, the Netherlands) (peak wavelength 311 nm) delivered on successive days (0.48, 0.52 and 0.57 J/cm<sup>2</sup>, respectively) to the same area of skin (10 cm<sup>2</sup>) on the right lower back. These exposures are about 80% of the median minimal erythema dose on the back of white-skinned subjects (8).

Skin colour was measured on the back of each volunteer using a portable reflectance instrument (Minolta Chromameter CR300, Osaka, Japan) using the *L\*a\*b\** system (Commission Internationale de l'Eclairage, <http://www.cie.co.at/cie/>), where the *L\** axis is of principal interest in terms of a pigmentary response (9), with higher values representing paler skin.

We have previously determined (10) that the time course for pigmentation is maximal between 1 and 2 weeks, so 10 days after the final irradiation, suction blisters were formed and removed from unirradiated and irradiated areas of the lower back of the healthy volunteers, and from the vitiliginous areas of the (unirradiated) subjects with vitiligo. The excised epidermis (1 cm diameter) was suspended in physiological-buffered saline in a quartz cell to eliminate dehydration of the sample (11). The quartz cell was then placed in the optical path of a Fluoromax-3 spectrometer (HORIBA Jobin Yvon Ltd, London, UK) operated in the synchronous scanning mode, and directly in front of the entrance aperture of an integrating sphere in order to collect all forward transmitted and scattered radiation. The spectral transmittance (relative to a cell containing just saline) was obtained over the spectral region 250–400 nm in 1 nm steps, and the spectral absorbance derived as the logarithm (to base 10) of the reciprocal of spectral transmittance.

#### Mathematical model

The fundamental metric is the spectral absorbance,  $A(\lambda)$ , at wavelength  $\lambda$  nm of excised epidermis from 250 to 400 nm. Although a rigorous mathematical description of the optical properties of the epidermis presents a formidable challenge (12), the absorbance of a sample of epidermis can be approximated as the sum of the individual absorbance of the constituent chromophores (13) of the sample and that because of scattering, and is given as

$$A(\lambda) = A(\lambda)_{\text{biomolecules}} + A(\lambda)_{\text{melanin}} + S(\lambda)$$

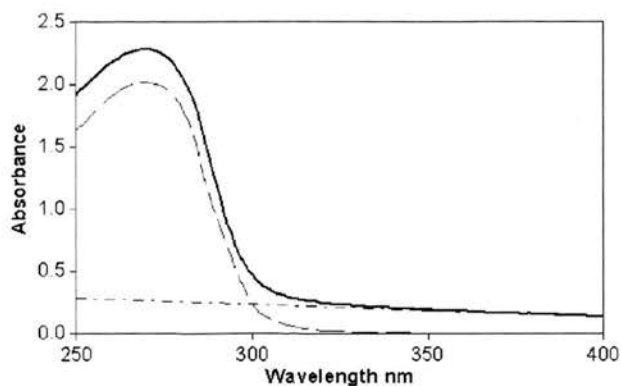


Fig. 1. The mean absorbance profile of excised, unirradiated epidermis obtained from the cohort of eight subjects with vitiligo (solid curve), the derived component because of scattering (dot-dash line) and the residual component because of absorption by amino acids and nucleic acids (dashed curve).

$A(\lambda)_{\text{biomolecules}}$  is the absorbance due to the major biomolecules of amino acids and nucleic acids, and is only significant at wavelengths less than about 310 nm;  $A(\lambda)_{\text{melanin}}$  is the absorbance due to melanin; and  $S(\lambda)$  is the attenuation due to scattering within the epidermis.

In vitiliginous skin, melanin is absent and so the scattering function for the mean absorbance profile of excised, unirradiated epidermis obtained from the cohort of eight subjects with vitiligo can be obtained by linear regression in the wavelength interval 360–400 nm (where absorption by biomolecules is negligible), and extrapolating to shorter wavelengths. Subtracting this function from the mean absorbance profile results in the absorbance due to amino acids and nucleic acids alone (dashed line; Fig. 1).

The solid line in Fig. 2 shows the mean absorbance profile of excised, unirradiated epidermis obtained from the cohort of 16 Caucasian subjects. Absorption due to melanin can be approximated by a Gaussian function peaking at 310 nm (14). Fitting this function by regression analysis to the solid line in Fig. 2 between 330 and 400 nm and subtracting the fitted curve from the total absorbance results in the absorbance due to amino acids and nucleic acids alone (dashed line; Fig. 2).

The component of the mean absorbance profile of excised, unirradiated, Caucasian epidermis due to scattering is simply the scattering function obtained for vitiliginous skin multiplied by the ratio of areas under the curves between 250 and 400 nm of the absorption profiles due to the biomolecules only for the Caucasian and vitiliginous epidermis (dashed

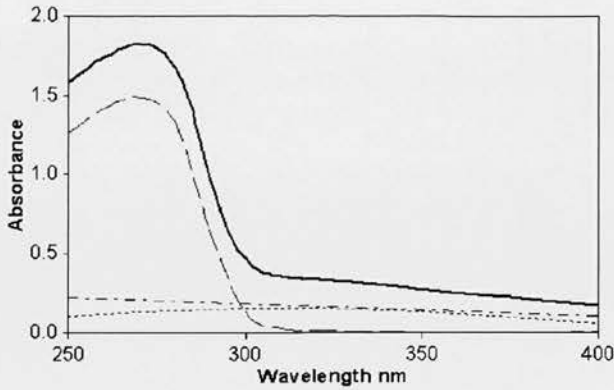


Fig. 2. The mean absorbance profile of excised, unirradiated epidermis obtained from the cohort of 16 Caucasian subjects (solid curve), and the derived components because of scattering (dot-dash line), absorption by amino acids and nucleic acids (dashed curve), and absorption because of melanin (dotted curve).

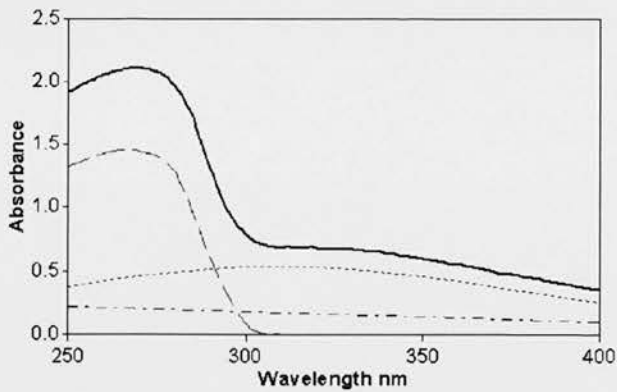


Fig. 3. The absorbance of a sample of excised epidermis obtained from the unirradiated site of an Asian subject (solid curve), and the derived components because of scattering (dot-dash line), absorption by amino acids and nucleic acids (dashed curve), and absorption because of melanin (dotted curve).

curves; Figs 2 and 1, respectively), as this ratio is equal to that of the equivalent thickness of the two samples. The resultant mean scattering function for unirradiated, Caucasian epidermis (referred to henceforth as the *reference sample*) is shown by the dot-dash line in Fig. 2.

Finally, the scattering function is subtracted from the total absorbance (solid line), which in the spectral region above about 330 nm leaves absorption due solely to melanin. Fitting these residual data by a Gaussian function (peaking at 310 nm) between 330 and 400 nm yields the dotted line in Fig. 2.

The sequence of steps described above was repeated for each sample of irradiated and unirradiated epidermis taken from each of the 20 volunteers. An example of the components of total absorbance for a given sample (in this case, unirradiated Asian skin) is shown in Fig. 3. The thickness of this excised epidermal sample relative to the reference sample is equal to the areas under the curves between 250 and 400 nm of the absorption profiles due to amino acids and nucleic acids only (dashed curves; Figs 3 and 2, respectively). The melanin content of the sample relative to the reference sample is equal to the ratio of areas under the curves between 250 and 400 nm of the absorption profiles due to melanin only (dotted curves; Figs 3 and 2, respectively). The density of melanosomes of the sample relative to the reference sample is now obtained as the ratio of relative melanin content to relative thickness.

## Results

The association between total melanin content of each unirradiated sample relative to the reference sample is shown in Fig. 4 as a function of skin colour (expressed by  $L^*$ ) measured in the unirradiated skin of each of the 20 volunteers. Grouped regression analysis applied to the datasets for Asian and Caucasian volunteers showed a highly significant common slope indicated by the solid line ( $P < 0.001$ ), with no significant differences between the slopes ( $P > 0.1$ ).

Figure 5 illustrates that with the UV irradiation scheme used in the present study, there was no change in the mean thickness of Asian skin pre- and post-irradiation, unlike that seen in Caucasian epidermis where the mean thickness following irradiation increased by 30% ( $P = 0.0015$ ; paired  $t$ -test).

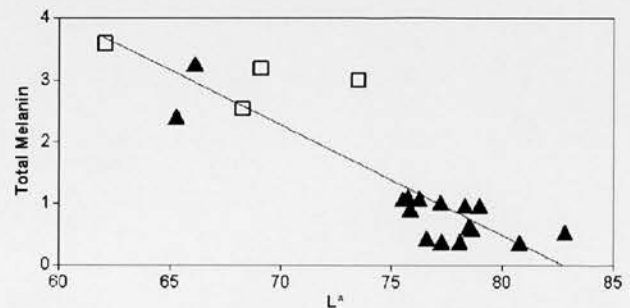


Fig. 4. The total melanin content of each unirradiated sample relative to the reference sample is plotted against skin colour (expressed by  $L^*$ ) measured in the unirradiated skin of each of the 20 volunteers. Asian skin is represented by open squares and Caucasian skin by solid triangles.



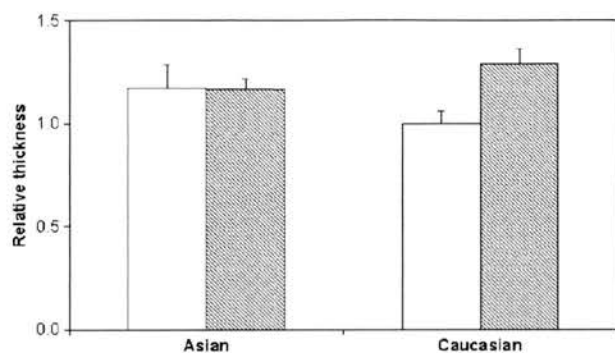


Fig. 5. Mean thickness of Asian and Caucasian skin pre-irradiation (open blocks) and post-irradiation (hashed blocks). The errors bars represent 1 standard error of the mean.

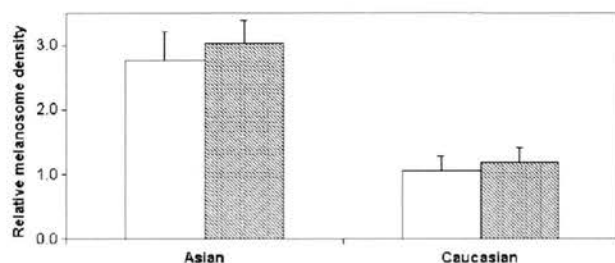


Fig. 6. Mean density of melanosomes of Asian and Caucasian skin pre-irradiation (open blocks) and post-irradiation (hashed blocks). The errors bars represent 1 standard error of the mean.

Not surprisingly Asian skin showed a higher baseline density of melanosomes than Caucasian skin (Fig. 6). In terms of changes in pigmentation, both Asian and Caucasian skin exhibited a small increase in the mean density of melanosomes between pre- and post-irradiated Caucasian skin (Fig. 6), although the effect was not statistically significant in either case ( $P = 0.1$  in both cases; paired *t*-test).

## Discussion

We have described a mathematical model for explaining the spectral absorbance of excised human epidermis based on the absorbance of constituent chromophores. The model was applied to spectral absorbance data measured on samples of epidermis excised from pre-irradiated skin and from skin obtained following UV irradiation on 3 successive days at doses sufficient to result in a minimal inflammatory response. We found that in Asian skin there was only a mild photoadaptive response, principally by a small increase in pigmentation. On the other hand, the significant adaptive response in Caucasian skin was

through hyperplasia of the epidermis, with tanning contributing only to a much smaller degree.

These results are in keeping with our earlier study (10) in that we found little relation between constitutive pigment and facultative pigmentation, with both Asians and Caucasians exhibiting similar small increases in tanning to 3 days of UV irradiation that would be equivalent, in terms of erythral response, to about 20 min of summer sunbathing each day around midday.

Both pigmentation and non-pigmentary processes contribute to the development of photoadaptation yet in our previous work we could not assay this latter process directly, merely overall photoadaptation by the use of repeated irradiation and subsequent challenge (10). However, the present study has enabled us to study independently the pigmentary and non-pigmentary pathways and has shown that in those people with a lower degree of constitutive pigment, the primary mechanism of photoadaptation is via the non-pigmentary route.

## Acknowledgement

We thank the Wellcome Trust for supporting this work.

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*Corresponding author:*

Prof. Brian Diffey  
Regional Medical Physics Department  
Newcastle General Hospital  
Newcastle upon Tyne NE4 6BE  
UK  
Tel: +44 191 273 1577  
Fax: +44 191 226 0907  
e-mail: b.l.diffey@ncl.ac.uk

# Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation

Alison Hennessy<sup>1</sup>, Carol Oh<sup>1</sup>, Brian Diffey<sup>2</sup>, Kazumasa Wakamatsu<sup>3</sup>, Shosuke Ito<sup>3</sup> and Jonathan Rees<sup>1,\*</sup>

<sup>1</sup>Department of Dermatology, University of Edinburgh, Edinburgh, UK

<sup>2</sup>Regional Medical Physics Department, Newcastle General Hospital, Newcastle NE46BE, UK

<sup>3</sup>Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake, Aichi 470-1192, Japan

\*Address correspondence to Jonathan Rees, e-mail: jonathan.rees@ed.ac.uk

## Summary

**Pheomelanin is widely thought to be causally related to susceptibility to the harmful effects of ultraviolet radiation: epidemiological studies show that those with a higher ratio of pheomelanin to eumelanin in hair have higher rates of melanoma, and work in mouse and cell culture shows that pheomelanin generates excess free radicals after UVR exposure. By contrast, based on measurements of eumelanin and pheomelanin in human skin, before and following irradiation, we now report that both pheomelanin and eumelanin are positively related to skin colour, and by inference, inversely with cancer susceptibility. The ratio of melanin classes is similar in people with widely different cancer rates and UVR sensitivity. Although our numbers are small, our results extend previous work in man, and lead us to speculate that factors other than the amount of pheomelanin may be important in determining UVR susceptibility in persons with red hair.**

Key words: eumelanin/pheomelanin/UVB/epidermis

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## Introduction

Ecological associations suggest that melanin is protective against the development of most forms of skin cancer (Urbach, 1999). In general, persons with the greatest constitutive pigment show the lowest rates of

non-melanoma and melanoma skin cancer, and the degree of skin pigmentation is inversely proportional to, and predicts, the amount of erythema that follows acute ultraviolet radiation exposure (UVR) (Ha et al., 2003; Wagner et al., 2002b).

Melanin is a complex mixture of biopolymers derived from tyrosine, produced in melanocytes and passed to surrounding keratinocytes. Melanin absorbs UVR across a wide spectrum with the highest absorption in the shorter wavelengths that are most associated with DNA damage from UVR (Young, 1997). Chemical elucidation of the structure of melanin is difficult because it represents a range of biopolymers, and extraction damages primary structure. Nonetheless, two broad classes of melanins are commonly the basis for assay; eumelanin which is brown and black, and pheomelanin which is red or yellow (Wakamatsu and Ito, 2002). The optical properties of these melanin types in the UV range are similar (Sarna, 1992). In mouse and human hair, the relative amounts of eumelanin and pheomelanin are under genetic control, playing with the MC1R a key regulatory role (Naysmith et al., 2004; Rees, 2003). Persons with red hair are likely to harbour one or two diminished function alleles at the MC1R, and their hair is characterized by a high ratio of pheomelanin to eumelanin (Naysmith et al., 2004). This switching of the relative amounts of the two melanin classes has been extensively studied in the mouse (Lamoreux et al., 2001; Ozeki et al., 1995).

Although interfollicular melanin (that is melanin present within the epidermis but outwith hair follicles) appears to be protective against skin cancer and UVR induced burning, the role of the different classes of melanin, eumelanin versus pheomelanin, is still debated. It is widely stated (Chin, 2003) that pheomelanin is a risk factor for skin cancer. This claim is based on two arguments, the association between persons with red hair and skin cancers (Rees, 2002), and the fact that under some conditions pheomelanin is a photosensitizer which generates active oxygen species upon UV irradiation (Hill and Hill, 2000). In support of the latter, Takeuchi (Takeuchi et al., 2004) recently showed, in the mouse hair follicle, that pheomelanin is associated with higher rates of TUNEL positive cells following UV irradiation.

There have been few measurements of melanin types in human skin (Hunt et al., 1995; Tadokoro et al., 2003; Thody et al., 1991; Tobin et al., 1994), compared with work in the mouse, cell culture or human hair (Ito and

Wakamatsu, 2003). Previous studies in man exploring the biological significance of pheomelanin in relation to ultraviolet sensitivity, notably those from the Naples group, have studied hair rather than skin melanins (Vincensi et al., 1998; Zanetti et al., 2001). In the present paper, we present data on eumelanin and pheomelanin, measured using standard HPLC assays, before and after UV irradiation. Our results do not of themselves clearly support a harmful role for pheomelanin in mediating acute damage, and suggest that current views of the relation between melanin, ethnic skin phenotype, and sensitivity to UVR are inadequate.

## Results and discussion

Figure 1 shows the eumelanin and pheomelanin concentrations plotted against constitutive skin colour for the total dataset. The unirradiated and irradiated data are linked for given individuals, with the unirradiated data being on the left hand side of the linked pair. Data are ranked on the X-axis by skin colour, measured as  $L^*$ .

Eumelanin levels in Asian skins increased from 4668 ng/mg (SEM 609) to 7212 ng/mg (SEM 1094) ( $P = 0.03$ ) on irradiation, while North European eumelanin levels increased from 1774 ng/mg (SEM 465) to 2229 ng/mg (SEM 476) ( $P = 0.01$ ). Pheomelanin levels in Asian skins increased from 290 ng/mg (SEM 77) to 462 ng/mg (SEM 129) ( $P = 0.04$ ), and in Northern European skins from 123 ng/mg (SEM 26) to 145 ng/mg (SEM 21) ( $P = 0.06$ ). All except three of the volunteers showed an increase in both types of melanin on irradiation. Because melanin is expressed per unit weight of epidermis, and epidermal thickness increases after irradiation, the reported values will underestimate melanin absorption by irradiated epidermis, and this fact may also account for the lowered melanin concentrations seen in these three persons.

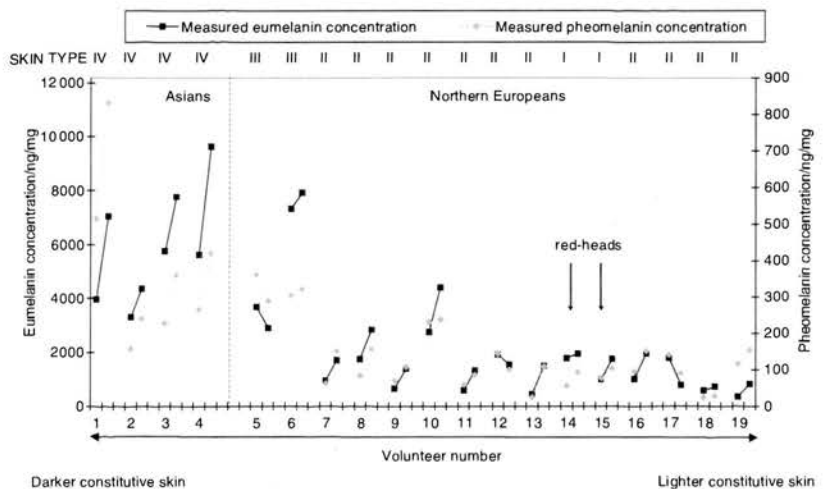
There was a marked correlation between eumelanin and pheomelanin in both constitutive skin ( $R^2 = 0.54$ )

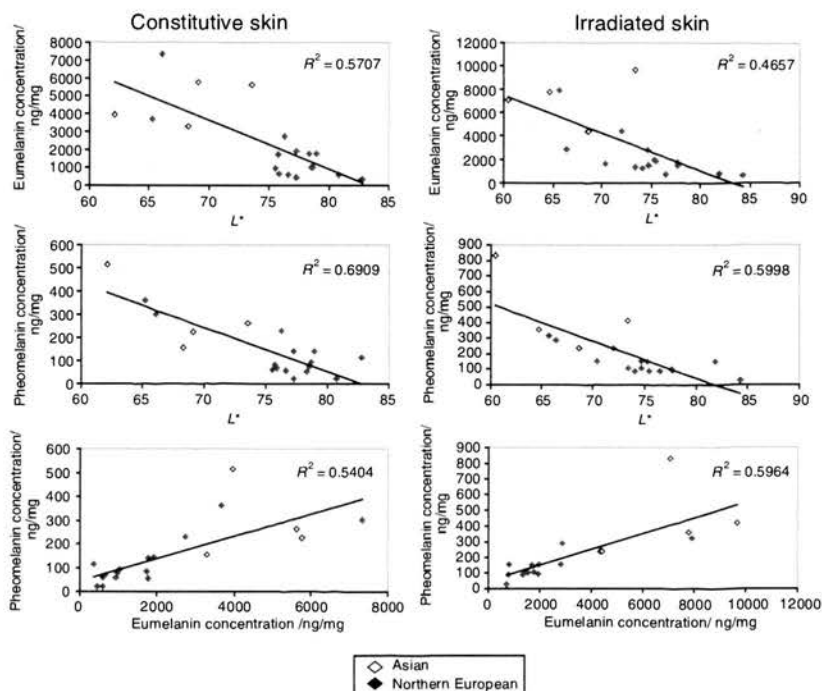
and irradiated skin ( $R^2 = 0.60$ ), and both eumelanin and pheomelanin were correlated with skin colour measured on the  $L$  scale (Figure 2) in constitutive skin ( $R^2 = 0.57$  and  $R^2 = 0.69$  for eumelanin and pheomelanin, respectively) and irradiated skin ( $R^2 = 0.47$  and  $R^2 = 0.60$  for eumelanin and pheomelanin, respectively). In contrast to earlier work the eumelanin : pheomelanin ratio appeared similar before and after irradiation for Asian skin [from 18.8 (SEM 3.8) to 17.9 (SEM 3.3) ( $P = 0.5$ )] and for Northern European skin [from 15.5 (SEM 7.61) to 15.4 (SEM 1.5) ( $P = 0.9$ )]. For constitutive pigment, we observed a non-significant difference in eumelanin : pheomelanin ratio of 18.8 (SEM 3.8) for Asian skin compared with 15.5 (SEM 7.61) for Northern European skin ( $P = 0.45$ ). Our results provide little support for large changes in the ratio of eumelanin to pheomelanin between different pigmentary phenotypes or before and after irradiation, and suggest that the presence of pheomelanin alone, or the ratio of eumelanin to pheomelanin is unlikely to explain known differences in UV sensitivity between persons of different skin colours.

There are some important differences between our methods and those used by earlier workers. We used the same irradiation schedule in all our subjects, rather than using a dose based on minimal erythema dose (Tobin et al., 1994). We believe our approach to be more appropriate. Second, all our melanin estimates were from the same body site, while the results of Hunt et al. (1995) were confounded by body site in that all their samples from darker skinned persons were fore-skins, whereas in Northern Europeans, a variety of sun exposed and sun protected sites were studied. Site variation in pigment and UVR sensitivity is large (Waterston et al., 2004). Finally, there have been refinements in the assays for pheomelanin (Kolb et al., 1997; Wakamatsu et al., 2002).

Our sample size, although bigger than that of previous studies, is still small, but the use of objective skin colour, measured on a continuous scale, rather than skin

**Figure 1.** Measured eumelanin and pheomelanin concentrations pre- and post-UVB irradiation. Unirradiated and irradiated data are linked for given individuals, with the unirradiated data being on the left hand side of the linked pair. Data are ranked on the X-axis by skin colour, measured as  $L^*$ . Fitzpatrick skin type classifications are also given.





**Figure 2.** Correlations between assayed eumelanin concentrations, pheomelanin concentrations and skin colour measured as  $L^*$  for Asian and Northern European skin types. Asian skin types are represented by hollow diamonds, Northern Europeans by solid diamonds.

type groupings, as Figures 1 and 2 show, lends some robustness to our results. It remains possible that the present chemical assays of pheomelanin are not capturing some important qualitative element associated with sensitivity to ultraviolet radiation. Clearly a larger study incorporating measures of acute UVR sensitivity is warranted, but we believe our results confirm and extend earlier findings. Higher levels of pheomelanin are found in those who are least, not most sensitive to UVR, and the melanin type switch, under the control of the MC1R may not be as critical for skin pigmentation as compared with hair pigment (Naysmith et al., 2004).

## Materials and methods

Twenty healthy volunteers were recruited from the Edinburgh area, and comprised four Asians and sixteen Northern Europeans. Three of the Asian volunteers were from South East Asia, and the other from India. All of the Northern European volunteers were from the UK. Three exposures of UV radiation from a Philips type TL-01 lamp (peak wavelength 311 nm) were delivered on successive days (0.48, 0.52 and 0.57 J/cm<sup>2</sup>, respectively) to the same area of skin (10 cm<sup>2</sup>) on the right lower back. These exposures are about 80% of the average minimal erythema dose on the back of white-skinned subjects (Gordon et al., 1998). We have previously determined (Oh et al., 2004) that the time course for pigmentation is maximal between 1 and 2 weeks, and 13 d after the final irradiation, suction blisters were formed and removed from unirradiated and irradiated areas of the lower back of the healthy volunteers.

The two classes of melanin were quantified by chemical degradation of eumelanin by acidic permanganate oxidation to form pyrrole-2,3,5-tricarboxylic acid (PTCA), and hydriodic acid reductive hydrolysis of pheomelanin to form 4-amino-3-hydroxyphenylalanine (4-AHP). Concentrations of these degradation products were then

determined using HPLC assays, and converted to eumelanin and pheomelanin concentrations by multiplying by factors of 160 and nine respectively (Wakamatsu and Ito, 2002).

Skin colour was measured on the back of each volunteer using a portable reflectance instrument (Minolta Chromameter CR300, Osaka, Japan) using the  $L^*a^*b^*$  system (Commission Internationale de l'Eclairage, <http://www.cie.co.at/cie/>), where the  $L^*$  axis is of principal interest in terms of a pigmentary response (Wagner et al., 2002a), with higher values representing paler skin.

Groups are represented using the mean and standard error of the mean (SEM) and were compared using paired or unpaired t-tests as appropriate. Linear regressions and  $r^2$  figures are quoted. Statistical analyses were carried out using Microsoft Excel.

## Acknowledgements

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## Ultraviolet Radiation Sensitivity in Vitiligo and Adjacent Normal Skin

Carol Oh, Alison Hennessy and Jonathan L. Rees

Department of Dermatology, The University of Edinburgh, Room 4.018, First Floor, The Lauriston Building, Lauriston Place, Edinburgh, EH3 9HA, UK.

E-mail: jonathan.rees@ed.ac.uk

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Sir,

We were fascinated to read the paper by Caron-Schreinemachers and colleagues (1) in which they showed, by elicitation of the minimal erythema dose (MED) on areas of vitiliginous skin, that ultraviolet radiation (UVR) sensitivity varied with total body skin type even in skin without pigment. We have been interested in similar issues and present our experimental data below. Although our patient series is much smaller we believe our results are germane to their work.

We studied seven persons with a range of skin types (2–5) and body-sites and, unlike Caron-Schreinemachers et al., assessed blood flow with a contact-Doppler after irradiation with three doses of UVB (28, 56, and 112 mJ/cm<sup>2</sup>, from a xenon arc lamp with an interference filter) on each area of vitiligo, and on the immediately adjacent normal skin. On all these individuals, we also estimated epidermal thickness by the use of transmission spectrophotometry on blister roofs for both the area of vitiligo and the adjacent skin (experimental details for this method are provided in our recent publication (2)).

The results were computed as the arithmetic difference between the blood flow in the area of vitiligo

and the adjacent area of skin for each of the three doses of radiation administered (Fig. 1). It can be seen that the difference between the area of vitiligo and normal skin varies with dose, i.e. the difference attributable to pigmentation is not constant but depends on the dose of UVR administered. Alternatively, if the ratio of vitiligo to normal skin blood flux, akin to the use of the sun-protection factor, is examined, our data again show that the effect varies with dose: means (and standard deviation in brackets) in ascending order for the three doses are as follows; 1.3 (0.49), 2.1 (0.84), 3 (2.1). Although our data is based on a small number of individuals, our results are compatible with a body of literature that suggests that any measure of sun protection due to pigment is dependent on the dose of UVR used. Put another way, as indeed Westerhof et al. (3) was the first to show many years ago, the gradient of the UVR dose–response curve differs depending on pigmentary status.

Finally, our use of the difference in blood flow following UVR between vitiligo and normal skin, as a measure of the sun-protection factor afforded by pigmentation will underestimate the effects of pigmentation, because previous work, and our own recent work, shows that vitiligo skin epidermis is thicker than adjacent skin (4). For instance, for the present series of individuals, the vitiligo epidermis was 1.26 ( $\pm$  0.15 SEM) thicker (2).

Finally, we congratulate Caron-Schreinemachers and colleagues on the elegance and power of their clinical experiment.

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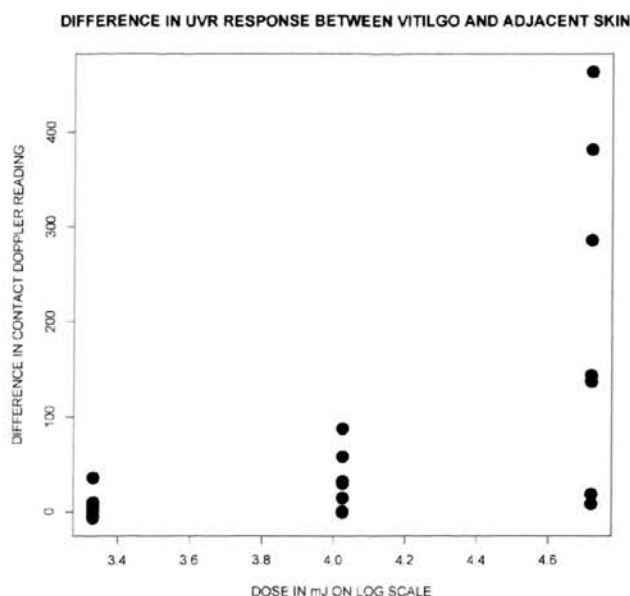


Fig. 1. The results, were computed as the arithmetic difference between the blood flow (contact Doppler reading) in the area of vitiligo and the adjacent area of skin for each of the three doses of radiation administered ( $n = 7$ ).